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BIOLOGICAL BULLETIN

ATTEMPTS TO CULTIVATE THE BACTEROIDS OF THE BLATTIDAE.[1]

MARSHALL HERTIG.

Since the discovery by Blochmann in 1887 of the bacteroids in the fat-body and eggs of cockroaches, these organisms have been studied by a number of investigators. The bacteroids are organisms very closely resembling bacteria in their morphology and staining reactions. They are found in the fat-body of roaches, filling the cytoplasm of certain cells, the bacteriocytes. They are also found forming a layer over the surface of ovarian eggs and in the yolk of the developing egg. They migrate from the yolk into the fat-cells of the half-grown embryo, where they are found in older embryos, nymphs and adults. The bacteroids have been found in all individuals of nine species of Blattidæ thus far studied, namely, *Blatella germanica*, *Blatta orientalis*, *Ectobia livida*, *Ectobia lapponica*, *Blaberus* sp., *Periplaneta americana*, *Periplaneta australasiæ*, *Parcoblatta virginica* and *Parcoblatta pennsylvanica*. No blattid is known to lack the bacteroids.

The morphology and behavior of the bacteroids in the host tissues are almost identical in all the species studied. The relation of the bacteroids to the host is not understood. The relationship may be merely one of harmless parasitism, there being maintained a delicately adjusted equilibrium between host and parasite, or it may be one of symbiosis, in which both bacteroids and their carriers derive some benefit from the association.

The striking morphological resemblance of the bacteroids to ordinary bacteria and the fact that they multiply within the body of the cockroach have led a number of workers to attempt

[1] Published with the approval of the Director, as Paper No. 246 of the Journal Series of the Minnesota Agricultural Experiment Station.

their culture in artificial media. Blochmann, their discoverer, was the first to attempt their culture in 1887. He employed a number of common laboratory media, as well as a special cockroach-infusion medium, but obtained only negative results. A few years later, Krassilschtschik ('89), according to Cholodkowsky ('91), also attempted the artificial culture of the blattid bacteroids in connection with his work on certain bacteria ("biophytes") found in the bodies of aphids. Though successful in the cultivation of the aphid bacteria, Krassilschtschik failed in his efforts to cultivate the blattid bacteroids. Forbes ('92), in his work on the bacteria normal to the digestive tract of the Hemiptera, stated that he had been unable to cultivate the bacteria found in the fat-bodies of cockroaches. However, Mercier ('06, '07) announced that he had succeeded in obtaining in pure culture on routine media, the bacteroids of *Blatta orientalis*. Tubes of bouillon were inoculated with material withdrawn by means of a sterile pipette from the oötheca, one end of which had been sterilized by flaming. In forty such experiments Mercier obtained pure cultures of an aërobic, motile spore-bearer, which he named *Bacillus cuenoti*. This organism, which grew luxuriantly on all the common laboratory media, resembled closely in cultural characteristics *Bacillus subtilis* and *B. mesentericus*, organisms commonly occurring as contaminators in cultures. Mercier satisfied himself that *Bacillus cuenoti* represented the cultural form of the bacteroids and made certain morphological correlations between the bacteroids and *Bacillus cuenoti*. Mercier obtained cultures from only the single stage, the oötheca, of but one species, *Blatta orientalis*. Javelly ('14) was unable to confirm Mercier's results. Cultures made by him according to Mercier's technique from a number of oöthecæ of *Blatta orientalis* and *Blatella germanica* remained sterile. Javelly concluded that *Bacillus cuenoti* was merely contamination, and that the bacteroids had not been cultivated in artificial media. Quite recently Glaser ('20) has reported the artifical culture of the bacteroids of *Periplaneta americana* and *Parcoblatta virginica*. The organisms obtained by him from both species are spirilla growing readily on routine media. These organisms differ in many important respects from *Bacillus cuenoti*.

In the work of the writer on the bacteroids of the Blattidæ, the results of which are in manuscript, a number of attempts have been made to cultivate artificially the bacteroids of *Blatella germanica*, *Blatta orientalis* and *Periplaneta americana*. The culture media employed include bouillon, agar, gelatin, sugar bouillon (under aërobic and anaërobic conditions), rabbit blood agar, peptone and ascitic fluid-bouillon. In obtaining the bacteroids for inoculation the following technique was employed. Oöthecæ were flamed at one end, a sterile capillary pipette thrust through such flamed surface to the opposite end of the oötheca, thus withdrawing material from within the unflamed as well as the flamed end. This material, which was shown by repeated examinations to contain bacteroids, was transferred to tubes of media. Cultures were also inoculated with bacteroids obtained from the fat-body and ovary. Segments of the abdomen were gently pulled apart by means of forceps, the intersegmental membrane being ruptured. By manipulating the forceps in pulling, one of the segments could be lifted away from the viscera, so that when the rupture occurred the segment would project like a shelf above the digestive tract with its adhering masses of fat-body. With a sterile platinum needle, portions of fat-body were "fished" from the under side of this shelf or from the surface of the digestive tract, care being taken not to touch the torn edge of the segment. By this method remarkably little contamination was encountered. Many cultures remained sterile, or yielded but a few scattered colonies on solid media.

From 20 oöthecæ of *Blatella germanica*, 25 cultures were made. 16 of these 25 remained sterile, while in the other nine there appeared a variety of organisms, none of which resembled the organisms obtained by either Mercier or Glaser. Comparable results were obtained in cultures from the fat-body and ovary. A number remained sterile, while in the other cultures appeared a variety of contaminating organisms. Attempts to cultivate the bacteroids of *Blatta orientalis* and *Periplaneta americana* yielded results entirely similar to those obtained with *Blatella germanica*.

In the endeavor to eliminate contamination entirely, there were made, in addition to the above, a number of cultures using

individual bacteroids which had been isolated by the pipette method of Barber ('14). Individual bacteroids obtained from *Blatta orientalis* were placed in hanging drops of peptone or ascitic fluid-bouillon. These bacteroids were charted and observed at frequent intervals. Ten hanging drops were made, the total number of bacteroids in these drops being about 100. No growth whatsoever of the bacteroids or of any other organism took place in these hanging drops. Single bacteria from a laboratory culture placed in control hanging drops multiplied readily. The results of the entire series of culture experiments on the three species of Blattidæ indicate that the bacteroids do not grow readily, if at all, on routine culture media.

It is thus seen that the culture studies of the various investigators have yielded conflicting results. The three earlier investigators, Blochmann, Krassilschtschik and Forbes, did not succeed in growing the bacteroids. Mercier obtained from *Blatta orientalis* an organism closely resembling a number of common contaminators. If the bacteroids could be grown on routine media as readily as *Bacillus cuenoti*, it should be possible to obtain this organism in all cultures inoculated with bacteroids from any stage in the life history of the roach, *i.e.*, from the fat-body, egg or embryo. The work of Javelly and the writer, in which many sterile cultures were obtained, and in which *Bacillus cuenoti* was not found, would seem to indicate that Mercier's organism was itself a contaminator. The results of Javelly and the writer further agree in indicating that the bacteroids of *Blatella germanica* as well as those of *Blatta orientalis* do not grow readily, if at all, in routine culture media.

In Glaser's work with *Periplaneta americana* and *Parcoblatta virginica*, he obtained from each species a spirillum, these two spirilla differing from each other only in minor cultural characteristics. They differ markedly, however, in both morphology and cultural characteristics from Mercier's *Bacillus cuenoti*. Glaser inoculated his cultures with bacteroids obtained from the fat-body of adults. The abdomen was washed with alcohol or a mixture of alcohol and corrosive sublimate, and dissected with sterile instruments. The portions of fat-body containing

bacteriocytes were transferred directly to media and incubated. In the writer's experiments with *Periplaneta americana*, three tubes of bouillon were inoculated with bacteroids from three oöthecæ, employing the technique described above. These three cultures remained sterile. A portion of the fat-body of a nymph was obtained by pulling apart the segments of the abdomen as described above, and a tube of bouillon inoculated. No growth occurred. In making a second transfer from this same nymph, the digestive tract was injured and contamination resulted. No other cultures were made from this species. While the number of cultures made from *Periplaneta americana* was limited, the results are seen to be in perfect agreement with those obtained by both Javelly and the writer for *Blatella* and *Blatta*. If the spirillum obtained by Glaser from *Periplaneta americana*, and which grows so readily in a great variety of routine media, actually represents the cultural form of the bacteroids of this species, it is difficult to account for the consistently sterile cultures obtained by the writer.

It was not stated by Glaser whether his technique yielded pure cultures of the spirilla in all cases, or whether contaminations were encountered. The subject of contamination was not discussed, either as concerns organisms in the cultures other than the spirilla or as to the possibility of the spirilla themselves being organisms other than the bacteroids. In view of the conflicting results of various investigators and the small size and filthy habits of the cockroach, the matter of possible contamination would seem to be a paramount consideration. In Mercier's work the flaming of the oötheca did not prevent contamination, and indeed, from his control experiments in which he obtained sterile cultures from the clear liquid bathing the eggs, it would appear that *Bacillus cuenoti* was obtained from within the egg. Javelly obtained contamination in cultures from the fat-body. In the writer's work, contamination was encountered in cultures from both oöthecæ and fat-body. It would seem impossible entirely to avoid contamination, except when employing single bacteroids isolated by the Barber or equivalent technique. The possibility of organisms other than

the bacteroids occurring within the tissues or body cavity of the roaches is not precluded, since all the writer's specimens of *Periplaneta americana* were heavily infected with a gregarine, *Diplocystis* sp., inhabiting the body cavity. Glaser did not mention any control experiments showing that the washing of the roach with alcohol or other reagents was successful in sterilizing the surface of the roach. It is conceivable that such reagent might fail to sterilize the areas where one segment overlaps another. In referring to the definite wanderings of the bacteroids in the body of the roaches and in the yolk of the egg, Glaser stated that the organisms in the species studied by him were motile. It is not clear whether motility of the bacteroids was observed directly, or whether the motility of the spirilla in cultures was referred to. In the writer's work many fresh preparations of the bacteroids obtained from five species of Blattidæ, including *Periplaneta americana*, were studied. No suggestion of motility was observed in any case. In view of the conflicting evidence and the technical difficulties involved, Glaser's evidence that the spirilla represent the bacteroids in culture would seem to be inadequate.

As concerns *Blatella germanica* and *Blatta orientalis*, the work of Javelly and the writer indicate quite definitely that the bacteroids do not grow on routine culture media, and that *Bacillus cuenoti* of Mercier is a contaminator. The entirely negative results of the writer in the culture of the bacteroids of *Periplaneta americana*, combined with the evidence from the other species, would seem to furnish strong presumptive evidence that the spirillum obtained by Glaser does not represent the cultural form of the bacteroids of this species.

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BIFURCATION IN THE EMBRYOS OF TUBIFEX.[1]

PAUL S. WELCH.

INTRODUCTION.

In the course of some experiments upon one of the common tubificids, *Tubifex tubifex* (Müll.), teratological phenomena were discovered in the early development which merit attention. Instances of bifurcation have been reported in collections of mature and semi-mature Oligochæta from natural environments, but always as great rarities and usually assumed to be the result of regeneration following some form of injury. A considerable number of bifid forms have also been produced in connection with regeneration experiments. Bifurcation as an embryonic phenomenon in Oligochæta has heretofore been known only from six records. According to Kleinenberg (1879, p. 219) and Vejdovsky (1888–1892, p. 250), Dugès (1828) observed and figured a double monster of "*Lumbricus trapezoides*," and Ratzel and Warschawsky (1868) described a similar abnormality in "*L. agricola*." Kleinenberg (1878; 1879, pp. 216–219) found bifurcating embryos not infrequent in the cocoons of "*Lumbricus trapezoides*." Vejdovsky (1888–1892, p. 252) found two such monsters in the cocoons of "*Lumbricus terrestris*," one instance in "*Allolobophora fœtida*," and a large number in "*Allolobophora trapezoides*." Korschelt (1904, pp. 257–258, Pl. 13, fig. 1) described and figured a bifid embryo taken from a cocoon of "*Allolobophora subrubicunda*." Weber (1917) studied the anatomy of twelve double embryos of "*Helodrilus caliginosus trapezoides*" although apparently others were found as indicated by the statement (p. 347) "Out of the 184 cocoons opened 57 contained only one individual, 101 two, and 25 eggs in various cleavage stages and 1 four embryos. Thirty-five of the 101 cases were in the form of monsters."

In order that the identity of the above-mentioned annelids be understood it is necessary to consider the present status of some

[1] Contribution from the Zoölogical Laboratory, University of Michigan.

of the old names just quoted. In connection with the following synonymy it should be noted that at present authorities on Lumbricidæ do not entirely agree as to whether *Helodrilus* or *Allolobophora* should stand as the name of that genus.

Helodrilus fœtidus (Savigny).

Allolobophora fœtida (Savigny).

Helodrilus caliginosus trapezoides (Dugès).

Lumbricus trapezoides Dugès.

Allolobophora trapezoides (Dugès).

Helodrilus subrubicundus (Eisen).

Allolobophora subrubicunda Eisen.

Lumbricus terrestris L., Müller.

Lumbricus agricola Hoffm.

It thus appears that several of these writers worked on the same species under different names. Failure to recognize this fact has evidently led Weber into the error (p. 339) of listing "*Lumbricus trapezoides*" and "*Allolobophora trapezoides*" as separate species. The same writer (p. 347), in discussing the difference of opinion expressed by Kleinenberg and Vejdovsky as to the origin of these monsters, suggests that such a difference "may be explained on the basis of the difference in the forms worked on by these two investigators," evidently regarding the species as different, although Vejdovsky (p. 11) states that the material was the same: "Aus meinen embryologischen Untersuchungen ergibt sich ferner, dass die genannte Art mit dem 'Lumbricus trapezoides,' deren Entwicklung *Kleinenberg* bearbeitete, identisch ist."

Only three of the above-mentioned reports were founded upon an appreciable number of bifid embryos, these three pertaining to the same species. The other records were based upon distinct rarities. All recorded instances fall within the Lumbricidæ, the highest family of the Oligochæta. The present paper presents the first account of embryonic bifurcation in one of the lower families (Tubificidæ) of the Oligochæta, these anomalies exhibiting remarkable frequency of occurrence and diversity of form.

Material.

The material on which this paper is based was identified by Professor Frank Smith as *Tubifex tubifex* (Müll.). Certain localities near Ann Arbor, Michigan, furnish an abundant supply of this annelid, and it has been possible, during the past three years, to obtain large quantities of sexually mature worms and cocoons from November until July either in the native environment or in cultures in the laboratory. Easy access to such an extensive supply of material has made possible the large number of examinations referred to in this paper.

Owing to the abundance of material it was often possible to secure cocoons easily by merely bringing masses of the mud into the laboratory, spreading it out in large shallow dishes, covering it with a small amount of water, allowing the finely divided matter to settle, and gently working over the material with a pipette. Fortunately the mud does not adhere to these cocoons and they will usually appear clean and distinct when uncovered. Furthermore, their color, which is usually of a very light orange, is such as to offer distinct contrast to the mud in which they occur and except for the rough resemblance to the small sand grains sometimes mingled with the mud, the recognition of the cocoons offers no particular difficulty. For securing large numbers of cocoons quickly the writer has employed the method of running quantities of pond and river margin materials through a set of graded sieves, the finest one having a mesh just small enough to retain the cocoons. The residue was then thoroughly washed by a gentle stream of water and examined under good light conditions. Undue handling of materials sometimes results in the loss by the cocoons of their ability to maintain perfectly clean external surfaces, fine particles tending to adhere and thus render cocoons difficult to detect.

Since this work was largely concerned with certain abnormalities in development the cocoons were handled as carefully as possible. That the sieve method of collection was not responsible for the production of abnormalities was demonstrated by comparing the materials so collected with those taken simultaneously from the same habitat by other methods.

Ordinarily, cocoons were carried through their development in the laboratory by merely transferring them to shallow dishes containing river or pond water. For convenience of examination such water was sometimes filtered, although this procedure is not always necessary since the finely divided matter will usually settle and has the advantage of serving as food material for the young worms after they emerge. Occasionally mould attacked the cocoons but its appearance was not frequent enough to offer serious difficulty.

The Cocoon.

Dixon (1915, pp. 85–86) has described briefly the cocoon of "*Tubifex rivulorum*," a form now regarded as identical with *Tubifex tubifex*. In practically all respects this description has been found correct. However, the color of the cocoons demands comment. Dixon describes them as "Whitish or greyish in colour and semi-transparent, but when viewed with the naked eye they appear opaque, but this is due to the eggs which they contain." In this work it has been found that the external appearance depends somewhat upon the degree of development of the eggs within. When first deposited they may have a whitish or creamy tinge but as development proceeds there is an increasing amount of yellow or orange, the maximum intensity being reached at the time when the young worms are ready to escape. At all stages the cocoon proper (the surrounding capsule) is colorless and transparent.

Excluding occasional malformed cocoons, the shape varies from ovoid to sub-spherical with two symmetrically formed, cylindrical, projecting necks, one at each end of the major axis. These necks bear an important relation to certain matters considered in this paper since they are the exclusive avenues of escape for the young worms. At the time of deposition these necks are completely filled by plugs of material somewhat similar in appearance to the remainder of the cocoon but distinctly less resistant since they are easily penetrated at the proper time, while in the hundreds of cocoons examined not a single one had been punctured or ruptured elsewhere although they were often subjected to considerable pressure by developing worms

within. The presence of the plugs indicates that no emergence has yet occurred. These plugs vary in form in different cocoons but are usually cone-shaped at both ends, the outer end extending beyond the distal margin of the neck and the inner end extending into the cavity of the cocoon.

The number of eggs included in a cocoon is subject to considerable variation. In more than five hundred cocoons studied the maximum was found to be seventeen and the minimum one. Cocoons containing only one egg were rare, but numbers above ten were rather common, although the general average was somewhat lower. Dixon (1915, p. 85) found a maximum of only thirteen or fourteen. The size of the cocoon is somewhat dependent upon the number of eggs present.

Development of the Eggs.

Owing to the transparency of the cocoon, development of the eggs can be followed with some ease. Two distinct steps occur in the progress from the newly formed egg to the free living condition of the resulting worm, namely, escape from the egg membrane, and subsequent emergence from the cocoon. Ordinarily, eggs "hatch" in a very few days by breaking the delicate, transparent egg membrane but escape from the cocoon is subject to surprising variation and since this phenomenon is directly concerned with the problems in hand, special attention was given to it. This variation occurs not only among different cocoons but also among eggs of the same cocoon. Emergence ordinarily occurs during the tenth to the twentieth day but not infrequently the sojourn within is much longer. Delayed emergences are usually, if not entirely, due to some inability to pass through the neck of the cocoon and usually results in the death of the worm, although occasionally escape is finally accomplished.

Dixon (1915, p. 86) was unable to find more than nine young developing from one cocoon and thought it probable that in those with an unusually large number of eggs certain eggs only develop, the others ultimately deteriorating. Attention was given to this matter and the occasional failure of certain eggs to develop was confirmed. However, more than nine worms

emerging from one cocoon were often observed, the maximum number noted being thirteen. Futhermore, there seems to be no way of predicting where the sterile eggs will occur. Apparently they do appear more frequently in the larger cocoons but are not exclusively confined to them. After the development has progressed for two or three days it is usually possible to distinguish eggs which will develop and those which will not since the latter retain for a time their initial appearance thus offering distinct contrast with the changing color and shape of the former.

These sterile eggs have at least two possible fates: (1) They seem to be consumed as food by the developing worms in the same cocoon whenever one or more have been delayed in their escape. Often every trace of such eggs is eventually obliterated. (2) Sometimes all of the developing worms escape early, leaving sterile eggs behind. Such cocoons soon swarm with microscopic organisms which facilitate the dissolution and disappearance of the contents.

Possibly these sterile eggs have something to do with enabling worms to live for long periods within the cocoon after completing their embryonic development. However, instances were observed where worms continued to live in that confinement long after all the granular egg contents had disappeared.

As mentioned before, the only avenues of escape from the cocoons are the two necks. After the plugs are removed the diameter of these necks is just sufficient to permit the average, normally formed individual to squeeze through. Departures from the normal body form, especially swellings or bifurcations, constitute very effective barriers and since, as will be shown later, abnormalities are common in *Tubifex tubifex*, many an individual never reaches the outside world. At the proper time for emergence, exploring movements, especially with the anterior end, are exhibited and when the aperture is finally discovered, efforts leading to escape are quickly exerted. Complete exit may require several minutes, even the greater part of an hour under some conditions. Exit seems to be more easily and more quickly made when the cocoon is surrounded by mud or debris than when deposited in water on the botton of a glass aquarium, this

advantage evidently being due, in part at least, to the anchorage which the emerging portion of the worm secures against the surrounding materials with its setæ.

Abnormalities.

In the course of this work several different types of abnormalities were found, all of which appeared early in development and were almost exclusively confined within cocoons since their form was such as to prevent their escape. The following forms were noted: (1) Whole body swollen and shorter than normal; (2) anterior region swollen, posterior region normal; (3) local swellings along the body; (4) bifurcations; and (5) combinations of bifurcations with 1, 2, and 3. Since this paper is concerned only with the fourth type, the other forms of abnormality will receive no further consideration.

Frequency of Bifurcation.

During the past three years a large number of these bifurcated worms have been found. While it is possible to examine a cocoon and detect bifid individuals previous to any emergence, they can be secured easily and in some quantity by allowing cocoons to develop under as favorable laboratory conditions as possible until most or all of the normal individuals have escaped. Since the abnormalities only very rarely escape they are left behind in the cocoons and are thus easily collected at a later time. Records based upon more than five hundred cocoons indicate that about twenty percent yielded bifurcated individuals.

The maximum number of bifid individuals found in a cocoon was three, one or two being more common. Thus far no relation between the number of eggs per cocoon and the number of bifurcations has been detected, the latter being about as frequent in cocoons with few eggs as in those containing the larger numbers.

Forms of Bifurcation.

The diversity of form of these bifurcations is striking and demands some notice here. While an occasional irregularity was difficult to interpret, most individuals could be classified

according to the following analysis, all of the types specified being represented. It should be stated that this analysis is based upon observations on the activities and external features of the living anomalies.

A. Either anterior or posterior extremity bifid—
  1. Bifurcation simple—
    a. Branches equal.
    b. Branches unequal.
  2. Bifurcation compound—
    a. Plane of bifurcation—
      (1) Secondary bifurcation in same plane as primary.
      (2) Secondary bifurcation at right angles to primary.
    b. Equality of bifurcation—
      (1) Parts of primary bifurcation equal; secondary equal or unequal.
      (2) Parts of primary bifurcation unequal, secondary equal or unequal.

B. Both anterior and posterior extremities bifid—
  1. Primary bifurcations in same plane.
  2. Primary bifurcations in different planes.
  3. Either or both bifurcations compound.

In the writer's records anterior bifurcation with normal posterior end occurs about twice as frequently as a posterior bifurcation with normal anterior end. Furthermore, individuals with both ends bifid are almost as frequent as anterior ones. One series of one hundred and ten records taken at random shows the following proportions: forty-five anterior, twenty-four posterior, and forty-one combined anterior and posterior bifurcations. Other records yield somewhat similar results.

In most instances the recognition of compound bifurcations involved no difficulty. Occasionally, however, a specimen appeared which seemed at first sight to be some form of trifurcation, but careful examination demonstrated that some at least were really compound bifurcations; others, however, could not be definitely classified by external examination. Compound bifurcations are not uncommon and a considerable number were observed during the progress of this work.

The length of the duplicated parts is a matter of considerable variation and the only general relation discovered is that the longer ones occur on the posterior end. Up to date, the writer has not found an anterior bifurcation to exceed six somites, while posterior ones may include seventeen somites. Instances were observed in which the posterior branches constituted more than one half the total length of the body. Many anterior bifurcations are only one somite in extent, two and three being also common. Posterior bifurcations may be short, however, comprising but one somite. A trunk portion of several somites extent is always present, no instances having been seen of such narrow connection of duplicated parts as described by Weber (1917) for *Helodrilus caliginosus trapezoides*.

Emergence of Bifurcate Individuals.

Among approximately four thousand young worms just emerged from cocoons the writer found only ten which exhibited bifurcations. These were all alive and active. Three were slightly bifid both anteriorly and posteriorly; six had slight anterior bifurcations and normal posterior extremities; and one had a very slight anterior bifurcation and a very deep bifurcation of the posterior end.

The discovery of these ten free individuals indicates clearly that while emergence is possible it is really a rarity. Since nine had completely emerged before they were detected, their method of escape is unknown. The remaining one, caught in the act, was emerging backward, the normal posterior end going in advance. This form of escape appeared to be effective, although the bifurcated anterior end gave some trouble in traversing the cocoon neck. This observation suggests the possibility that the above-mentioned individuals with normal posterior ends emerged backwards. Added weight is given to this suggestion by the repeated observation that in the escape efforts of normal individuals the posterior ends were often projected through the cocoon neck. It seems that the escape of the specimen with shallow anterior bifurcation and very deep posterior one of necessity must have been with the anterior end in advance

since as will be shown later deep bifurcations present difficulties which are practically insuperable.

The difficulty of escape from the cocoon by a bifurcate individual is due to one or both of two features, namely, the increased bulk of the double end thus exceeding, ordinarily, the diameter of the neck passage, and the split nature of the end causing the frequent situation of one branch projecting through the neck and the other turned up inside the wall—a situation which is prohibitive of escape. Escape by working the way out backward has already been mentioned but that likewise appears impossible when the posterior end is deeply bifurcate. Even the most striking inequality of diameter in the arms of a bifurcation observed constitutes a serious obstacle to emergence. Both anterior and posterior extremities execute searching movements and instances have been found in which a worm with a deep posterior bifurcation had one posterior branch projected through one neck of the cocoon and the other through the opposite neck. Every kind of bifurcate individual manifests movements which in the normal animal would result in emergence and there seems to be no doubt whatever that failure to escape is due entirely to the abnormal body structure.

Viability in Cocoons.

Mention has already been made of the remarkable viability of some of these worms in cocoons and it should be stated in this connection that both bifurcate and non-bifurcate anomalies exhibit this phenomenon. The writer has records of sojourns within the cocoon for varying periods with ninety-eight days as a maximum and while in almost every case the individual untimately died without having escaped, a few of them (even in the record for ninety-eight days) finally emerged. Why escape could occur after so long a period is not known since the cocoon undergoes no observable change, but it is conceivable that such a form living for days after all the available food material apparently has been exhausted might, because of the continued state of inanition, become reduced somewhat in body size, this reduction permitting the animal to pass out through

the cocoon opening. Such body diminution is said to occur in certain other animals when subjected to a continued absence of food. Failure to emerge by non-bifurcate individuals was apparently due to abnormal proportions, this condition being attained before the individual was ready to leave the cocoon.

Rearing of Bifurcate Individuals.

Attempts were made to determine whether it is possible for bifurcate individuals to live and develop if assisted in escaping from their cocoons. In order to insure that they had reached a degree of development at least comparable with that of their normal fellows which were escaping and successfully assuming the free-living life, cocoons containing bifurcations were isolated and bifurcate individuals allowed to remain in the cocoons until most or all normal individuals had emerged. Then the former were dissected out with as great care as possible and introduced into a culture identical in composition and operation with the one in which the normal, newly emerged worms were thriving. Normal worms were used in this way as controls. Up to date the writer has been unable to rear a single bifurcation, although the controls were easily maintained. The reasons for this failure are not understood at present.

Absence in Natural Conditions.

The almost complete, if not wholly complete, absence of bifurcate individuals of *Tubifex tubifex* in nature is shown by the fact that in an examination of over 1,000 worms representing various degrees of maturity and immaturity and collected both from cultures and from native environments not a bifid form was found. The literature seems to contain only one record (Abel, 1902) of a bifid tubificid found in nature, but since similar results have been produced artificially in post-embryonic stages, there is no certainty that Abel's record has any significance in this connection since it might have resulted from an accidental injury. Evidently the cocoon constitutes a very efficient device which automatically eliminates the vast majority of abnormalities.

How universal this high frequency of bifurcation is in the development of *Tubifex tubifex* in all of the varied conditions under which it lives remains yet to be determined. Furthermore, no statement can be made at this time concerning the relation of bifid embryos to those extremely rare bifid oligochætes found in nature, although the available evidence leads one to suspect that the former do not survive and that the latter are the result of regeneration following injury.

Morphology.

Only a preliminary account of the internal structure of these anomalies, based upon serial sections of a few individuals, will be given at this time. All specimens studied thus far show the trunk portion of each worm to be a double region whose construction would be simulated by opposing intimately the cut surfaces of two similar ventral halves of normal embryos of the same degree of development. The branches of the bifurcate portion, however, lack this doubleness, being constructed on the plan of a normal individual. Evidence of the double character of the trunk portion is most readily exhibited in the setæ bundles, the nervous system, the nephridia and the principal blood vessels.

Sections through bifurcate portions of specimens show in each branch four setæ bundles arranged in the normal fashion with the component setæ so set and curved as to conform with those of normal specimens provided the nerve cord be interpreted as corresponding to the normal ventral one. In the trunk portion of the body eight bundles of setæ occur in sets of four, each group being the reverse of the other in arrangement and each being properly related to one of the two ventral nerve cords. Furthermore, the distribution of uncinate and capilliform setæ is as this interpretation would require.

Throughout the trunk portion two nerve cords, 180 degrees apart, extend without change to points where bifurcation appears. If the anterior end be unbranched, a mass of nervous tissue encircles the pharynx; if on the other hand it be branched each branch bears a cerebral mass and esophageal commissures.

If the bifurcation plane is frontal in position each nerve chain passes unchanged into each of the branches. Certain modifications of this arrangement occur if the plane of division be sagittal, chief among which is the swinging to one side or the other of each nerve cord.

Nephridia are sufficiently developed in these forms to be detected and one pair is normally related to each nerve cord in each somite, that is, two pairs per somite in the trunk portion. In the branches there is only one pair per somite.

In the bifurcate portions, the main vessels of the circulatory system occur in the normal position, but at the origin of the branches, the two dorsal vessels approach each other and are then laterally displaced by the union of the alimentary canals, one occupying a position to the right, the other to the left. A ventral vessel occurs just above each nerve cord.

It thus appears that at least some of these double embryos are of the type representing dorsal union, the type which Vejdovsky (1888–1892, p. 257) evidently referred to as "Die Doppelmissbindung, deren Individuen längs der Rückenseiten verwachsen," and which Weber (1917, p. 342) has also discussed. While none of the other types found by these authors in double embryos of certain Lumbricidæ have thus far been detected in *Tubifex tubifex* it is possible that some or all of them will appear when a more extensive study involving a much larger quantity of material is completed. Such a study is in progress and results will be reported in a subsequent paper. No attempt is made at present to account for the origin of these anomalies.

Summary

1. *Tubifex tubifex* (Müll.) deposits from one to seventeen eggs per cocoon, a few of which are often sterile.

2. Emergence of young worms from the cocoon is exclusively through two oppositely located apertures.

3. Various forms of abnormality commonly appear among the developing worms, chief among which are bifurcations of the body. Approximately twenty percent of the cocoons examined contained bifurcate embryos. This is the first report of bifur-

cation as an embryonic phenomenon in any of the lower famiies of the Oligochæta.

4. Diverse forms of bifurcation involving both extremities of the body occur in either simple or compound form.

5. Of more than four thousand recently emerged worms examined only ten bifid individuals were found. Any departure from normal body form involving increase in diameter constitutes an extremely effective barrier to escape from the cocoon and practically all of the numerous abnormalities are automatically eliminated.

6. While bifid worms may live for long periods of time imprisoned within the cocoons, emergence is a rarity.

7. Attempts to rear bifid individuals have thus far been unsuccessful.

8. An examination of more than one thousand worms from natural habitats and representing various stages of maturity yielded no bifid forms.

9. A preliminary morphological study, based principally on the setæ, the nervous system, the nephridia, and the circulatory system, shows the body region of these monsters to be double in composition while the bifurcate portions simulate the normal tubificid structure.

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OXYGEN CONCENTRATION AS A LIMITING FACTOR IN THE RESPIRATORY METABOLISM OF PLANARIA AGILIS.

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Living organisms have been conveniently grouped into obligate aërobes, facultative aërobes, facultative anaërobes and obligate anaërobes. The criterion for this classification is fundamentally the relation of organisms to the *concentration* of free oxygen. Free oxygen by its concentration effects on the metabolism acts as a limiting factor in determining animal and plant distribution. While the rôle of oxygen concentration in the life of lower plants and animal parasites has been known in a general way for a long time, yet the importance of differences in oxygen concentration as a factor determining the differences in distribution of many invertebrate animals, which we would ordinarily consider aërobic, has not received the emphasis which it deserves. The grouping of organisms into the above four groups is merely arbitrary, for from the small amount of data available it is clear that some organisms stand in an intermediate position between the above groups.

Since the rate of respiration is correlated to such conditions as state of nutrition, size and age, regeneration, cell division and irritability, it is logical to suppose that by comparing the effects of different oxygen concentrations in the different states of nutrition, age, regeneration and so on, valuable information might be obtained which would throw further light on the relation of respiration to this group of correlated processes.

In the present paper three questions will be considered in so far as they apply to *Planaria agilis*: (1) What is the quantitative relation between oxygen concentration and the rate of oxygen consumption? (2) What is the effect of the state of nutrition upon the relation of oxygen concentration to rate of oxygen

consumption? (3) What is the relation of oxygen concentration to the rate of carbon dioxide production and irritability in *Planaria agilis*, which ordinarily would be considered aërobic?

The Relation of Oxygen Concentration to the Rate of Oxygen Consumption.[1]

The change in rate of oxygen consumption by *Planaria agilis* while it is gradually consuming a given quantity of oxygen dissolved in a given volume of water in a stoppered bottle is shown in the curve in Fig. 1. The data for the curve were obtained as follows: Twenty-one bottles of equal volume were filled with

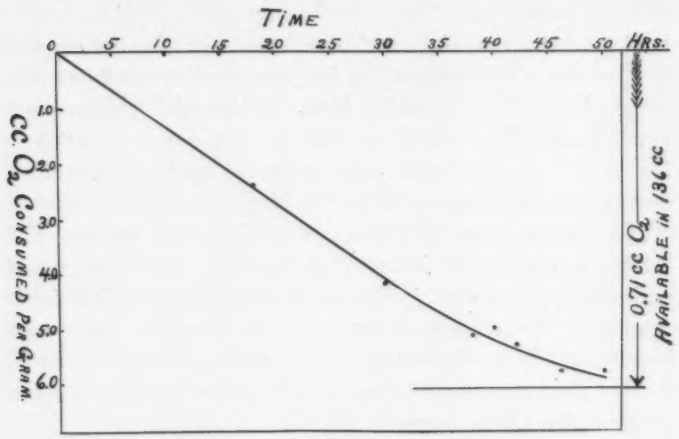

Fig. 1.

water containing the same amount of oxygen. Ten animals of equal size and the same history were placed in each bottle. At the end of different time periods as indicated in the curve three bottles were analyzed for oxygen content and the average taken as the value which is plotted on the ordinate. The animals had starved for over ten days previous to the experiment and therefore had practically a constant rate of respiratory metabolism as shown by Allen ('19, II.).

[1] The following is a part of certain studies on respiratory metabolism made possible by a grant from the research fund of the graduate school.

The animals which were used in the experiments reported in this paper were kept at 20° C. during the experiments, and for from two days to two weeks previous to each experiment in order to avoid any acclimation effects due to temperature. The curve shows that the rate of oxygen consumption was constant up to about thirty-eight hours, at which time the oxygen concentration was equal to about one fourth to one sixth of the oxygen concentration of air-saturated water at 20° C. When lower oxygen concentrations were approached the rate decreased. An unused residue of oxygen remained at the end of the experiment. This would have been quickly consumed if the original rate of oxidation had been maintained.

In this method of experiment the possibility that acclimation of the rate of oxygen consumption to lower oxygen concentrations occurs is not excluded. Therefore the following procedure was adopted. If the animals are subjected *suddenly* to definitely known low oxygen concentration and the time period during which they are left in this low oxygen concentration is made brief, then acclimation will probably be avoided. The procedure was as follows. Twenty-eight lots of worms with twenty worms in each lot were selected. All the animals were very closely alike in size, history, etc. The experiment was continued for three successive periods of three hours each. During the first period each one of the lots of animals was placed in a bottle containing air-saturated water. The rate of oxygen consumption per gram worm per hour for this period was determined. At the end of the first period the twenty-eight lots of animals were transferred directly into a second set of twenty-eight bottles containing different but known amounts of oxygen per 136 c.c. water. The animals were now left in these bottles for a second period of three hours. There were fourteen different known concentrations of oxygen. Two bottles were used for each concentration in order to increase the accuracy of the determinations. The fourteen different concentrations of oxygen were as follows: 0.029 c.c., 0.048 c.c., 0.085 c.c., 0.106 c.c., 0.127 c.c., 0.149 c.c., 0.168 c.c., 0.177 c.c., 0.215 c.c., 0.265 c.c., 0.280 c.c., 0.711 c.c., 1.420 c.c. and 1.840 c.c. per 136 c.c. of water.

In this series 0.711 c.c. of oxygen per 136 c.c. water represents the concentration at air saturation at 20° C. The different concentrations of oxygen were obtained by properly mixing known volumes of water of high oxygen content with oxygen-free water.

At the end of the second period of three hours, each lot of worms was again immediately transferred to a bottle containing air-saturated water and left for a third period of three hours. The rate of oxidation per gram per hour during this third period was again determined.[1]

The only results from this experiment with which we are concerned at this time are the rates of oxygen consumption per gram per hour during the *second* period in different concentrations of oxygen. These amounts of oxygen corresponding to the fourteen different concentrations of oxygen are represented by the points on the curve in Fig. 2. Each point is an average of the deter-

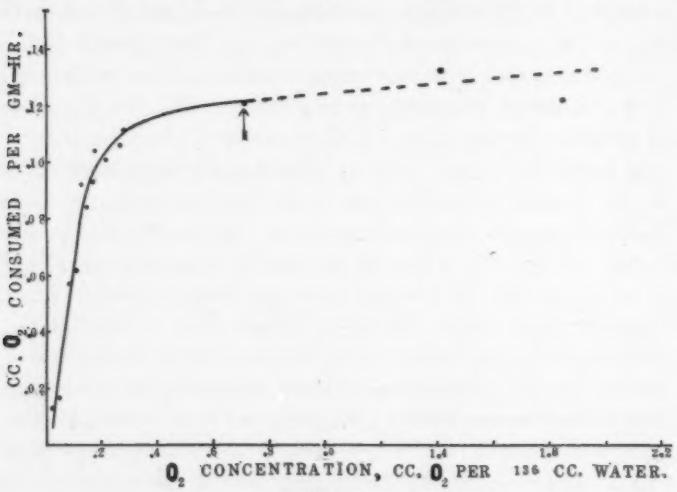

FIG. 2.

minations on two lots of worms. From the curve in Fig. 2 it will be seen that the oxygen concentration which begins definitely

[1] Many precautions and details of technique must be observed in such a relatively complex experiment so that the three-hour period was selected as most suitable. The writer is indebted to Miss Edna Wolf for valuable help in many of the experiments reported in this paper.

to act as a limiting factor on the rate of oxygen consumption lies somewhere around one third and one fourth of the concentration at air saturation, a little higher than that indicated by the curve in Fig. 1. Whether or not the respiratory mechanism of *Planaria* can actually adjust itself by maintaining to some extent a constant rate of oxygen absorption at oxygen concentrations below that which is limiting in such an experiment as the one given above, is an interesting question and will be referred to again.

The question arises as to whether or not *Planaria* can remove the last traces of dissolved oxygen. In general the impression obtained from many experiments of one type or another was, that the very last traces of oxygen could in general not be removed.[3]

The slope of the part of the curve shown by the interrupted line is the part which was left in some doubt in this experiment. Hence several separate tests were made on different lots of animals in order to determine more definitely whether high concentrations of oxygen do increase the rate of oxygen consumption. In each of the experiments two identical lots of animals were tested at air saturation and high oxygen concentration. The following are the results of three separate tests on different lots of worms.

	Concentration of Oxygen at Beginning. C.c. O_2 in 136 c.c. water.		Oxygen Consumed per Gram per 24 Hours.
Exp. I.	0.820		4.02
	1.996		4.38
Exp. II.	0.797	(Air Sat.)	2.92
	1.768		3.36
Exp. III.	1.320		3.88
	2.920		3.74

In general the results from several experiments indicate that there is a slightly higher rate of oxygen consumption at concentrations above air saturation than at air saturation. The approximate amount of this increase is indicated by the slope of the broken part of the curve. The experiment clearly shows that

[3] Winkler's method as ordinarily used is however not sufficiently accurate as a means of giving an absolute answer to this question, for iodine from potassium iodide is slowly set free by an excess of acid.

oxygen concentration may act as a limiting factor in the rate of oxygen consumption and shows also what are the quantitative relations.

This result is similar to those reported by Henze (10) for *Anemonia sulcata*, by Thunberg ('05) for *Limax* and *Lumbricus*, and in the leech as found in this laboratory by Miss Wolf. In a previous paper by Lund ('18) it was shown that the rate of oxygen consumption by *Paramecium caudatum* is practically entirely independent of the concentration of oxygen. This relation would be represented in Fig. 2 by a straight line drawn from some appropriate point on the y-axis parallel to the x-axis. Evidently the oxidation reactions in *Paramecium* and *Planaria* are quite different in certain respects. This is further shown by the fact that the inhibitory action of KNC on the rate of oxygen consumption in *Planaria* is very marked, as shown by Allen ('19, I.), while a similar inhibitory action on the oxygen consumption in *Paramecium caudatum* could not be found, Lund ('18, II.).[4]

It may well be that we shall be able to find in the results of studies on the relation of oxygen concentration to the rate of oxygen consumption, a key to the solution of the question as to why it is that the action of KNC upon *Planaria agilis* and *Paramecium caudatum* is different in character. Work is under way on this question.

It must be kept in mind that since we do not as yet have any accurate data on the magnitude of the diffusion coefficients of oxygen through the protoplasm of cells and different tissues, it is really impossible to say whether in such a case as *Planaria* for example, the decrease in the rate of oxygen consumption at lower oxygen concentrations is due to slow rate of diffusion of the oxygen into the interior of the animal, which rate is itself a function of the difference of oxygen pressure between the inside and outside of the animal, or whether the difference is actually due to an inherent difference in reaction velocity as expressed

[4] Various kinds of criticisms of these experiments have been made by Child and Hyman. A careful consideration of the objections offered has however entirely failed to disturb my confidence in the results as given in the experiments, and the correctness of the conclusions drawn from them. A more complete consideration of the criticisms with further experimental data is given elsewhere. Lund ('21.)

for example by the velocity constant in the equation for a bimolecular chemical reaction, Krogh ('19). In the absence of appropriate experimental data further discussion of this question is unprofitable.

Whatever may be the ultimate explanation or cause of the drop in the curve toward the origin of the axes, such curves for different animals nevertheless may turn out to be useful in interpreting the rôle of oxygen concentration as a factor in determining the distribution of the organism, and if further experiment can show that difference in concentration of oxygen between the outside and inside of the animal does not act as the limiting factor, but rather the concentration of oxygen as such in the cells, then such curves will be very important criteria for the differentiation of different types of respiratory metabolism among animals. A careful comparative study of, for example, the terrestrial, semi-aquatic, and mud-dwelling species of earthworms might throw further light on the question.

The Rate of Oxygen Consumption by Planaria in aërated Water After Living in Water of Low Oxygen Concentration.

Planaria agilis shows a characteristic increase in the rate of oxygen consumption after removal to aërated water from oxygen-free water. This is illustrated in Table I. The experiment was divided into three consecutive test periods. During the first period tests on the seven lots of animals were made in air-saturated water. After eight hours the lots were transferred to a low concentration of oxygen for periods of seven, nine and twelve hours, and at the end of these test periods they were transferred directly into aërated water and the rate of oxygen consumption per gram per hour determined for a third period of four hours. Table I. is a complete statement of the results.

It will be noted that during the third period there is an increase in rate over that in the first period varying from 49 to 85 per cent. There is no proportionality between this percentage increase and the length of time which the animals were subjected to the low oxygen concentration as one might at first expect.

TABLE I.

Planaria starved 26 days before experiment, and kept at 20° C. for 7 days before and during tests. 20 animals in each bottle.

Bottle.	Period I.—8 hours. Air-saturated Water. C.c. O_2 in 136 c.c. 0.713 0.715 0.715			Period II.—7–12 hours. Water with Low O_2 Content. C.c. O_2 in 136 c.c. 0.033 0.034 0.034			Period III.—4 hours. Air-saturated Water. C.c. O_2 in 136 c.c. 0.729 0.736 0.732		
	Weight of 20 Worms.	Total O_2 Consumed.	O_2 Consumed per gm. per 24 hours.	Length of Period.	Total O_2 Consumed.	O_2 Consumed per gm. per 24 hours.	Total O_2 Consumed.	O_2 Consumed per gm. per 24 hours.	Per Cent. Increase in Rate of O_2 Consumption.
	gms.	c.c.	c.c.	hrs.	c.c.	c.c.	c.c.	c.c.	
1	.167	.164	2.95	7	.012	.248	.123	4.41	49%
2	.165	.143	2.61	7	.017	.352	.132	4.77	82%
3	.165	.152	2.76	7	.014	.286	.140	5.08	84%
4	.164	.161	2.93	9	.026	.420	.147	5.36	82%
5	.163	.157	2.90	9	.026	.420	.138	5.10	75%
6	.161	.147	2.73	12	.026	.420	.137	5.08	85%
7	.163	.156	2.83	12	.026	.420	.126	4.64	63%

TABLE II.

Planaria starved 14 days before experiment. Temperature 20° C. 11 days before and during tests. Twenty animals in each bottle. Numbers in the table are averages of three bottles. Limit of experimental error 0.010 c.c. oxygen.

Bottles.	Total Weight of Worms in Grams.	Period I. 6 hours. Air-saturated Water. C.c. O_2 in 136 c.c. 0.720		Period II. C.c. O_2 in 136 c.c. Bottles 1 to 12, 0.029 Bottles 13 to 21, 0.062			Period III. 6 hours. Air-saturated Water. C.c. O_2 in 136 c.c. 0.734		
		Total O_2 Consumed.	O_2 Consumed per Gram per 24 Hours.	Length, Hrs.	Total O_2 Consumed.	O_2 Consumed per Gm. Hr.	Total O_2 Consumed.	O_2 Consumed per Gm. Hr.	Increase.
		c.c.	c.c.		c.c.	c.c.	c.c.	c.c.	
1, 2, 3....	0.569	0.42	2.95	1.5	(0.01 −)	0.2 −	0.52	3.67	24%
4, 5, 6....	0.608	0.41	2.71	3	0.02	0.25	0.57	3.79	36%
7, 8, 9....	0.567	0.43	3.04	4	0.04	0.42	0.56	3.99	31%
10, 11, 12	0.593	0.40	2.72	6	0.04+	0.31	0.55	3.69	35%
13, 14, 15	0.572	0.41	2.85	9	0.13	0.60	0.68	4.75	66%
16, 17, 18	0.560	0.45	3.19	16	0.15	0.41	0.74	5.31	66%
19, 20, 21	0.561	0.46	3.31	24	——	——	0.70	5.00	51%

To test further whether the length of time during which the animals lived in low oxygen concentration had any effect on the percentage increase during the third period, the results of an experiment in Table II. are given. The procedure was the same as that in the experiment, Table I., except that a larger number of bottles were used, with a greater range in the length of the second period, the latter varying from 1.5 hours to 24 hours. Roughly speaking, there is a slightly smaller average acceleration of the rate in the lots of worms subjected to low oxygen for the shorter periods than in the lots left in the low oxygen for the long periods. The difference in the increase is in general quite independent of the length of the period of low oxygen.

Comparison of the Rates of Oxygen Consumption in Air-Saturated Water by Starved and Fed Animals Immediately After Having Been Subjected to Low Concentration of Oxygen.

If we assume that the mechanism which determines the rate of oxygen consumption can undergo an adjustment when subjected to low oxygen concentration, in such a way as to compensate for the inhibitory effect of low oxygen on the rate of consumption, then, would it not be possible to test this assumption by comparing the acceleration, in rate of oxygen consumption, after life in low oxygen, of two lots of animals one of which has been previously subjected to repeated periods in low oxygen concentration, while the other has been manipulated mechanically in exactly the same way without subjection to low oxygen previous to the test?

The data in the columns under lot A and lot B in Table III. give the results of one test. Lot A was subjected to very low oxygen during two alternate periods of eight hours each, while lot B was manipulated similarly except that it was kept in aërated water. The average actual increase in rate of oxygen consumption by twenty animals during the third period is 1.08 c.c. oxygen per gram per 24 hours in lot A, while in lot B it is 1.54 c.c. oxygen per gram per 24 hours. The average increase is therefore 33 per cent. for lot A and 51 per cent. for lot B. A comparison of

TABLE III.

Planaria starved 7 days before experiment and kept at 20° C. 3 days before and during tests. Each lot of 80 worms was divided into four groups of 20 each for the tests.

	Lot A (80 worms). *Starving.*		Lot B (80 worms). *Starving.*	Lot C (80 worms). *Fed.*
1 Period 8 hrs.....	Low O_2 concentration.		Air-saturated water.	Air-saturated water.
2 Period 12 hrs.....	Air-saturated water.		Air-saturated water.	Air-saturated water.
3 Period 8 hrs.....	Low O_2 concentration.		Air-saturated water.	Air-saturated water.
4 Period 44 hrs.....	Air-saturated water.		Air-saturated water.	Air-saturated water. Fed liver at beginning of period.
	Bottle.	O_2 Consumed per Gram per 24 Hours.	O_2 Consumed per Gram per 24 Hours.	O_2 Consumed per Gram per 24 Hours.
Period I. 6 hours c.c. O_2 in 136 c.c. = 0.611		c.c.	c.c.	c.c.
	1	3.20	2.75	4.76
	2	3.30	3.09	4.89
	3	3.26	2.87	5.23
	4	3.31	3.36	4.69
	Avg.	3.27	3.01	4.89
Period II. 10 hours c.c. O_2 in 136 c.c. = 0.048	1	.37	.408	.42
	2	.37	.396	.43
	3	.37	.392	.42
	4	.40	.430	.38
	Avg.	.38	.40	.41
Period III. 6 hours c.c. O_2 in 136 c.c. = 0.672	1	4.14	4.61	6.15
	2	4.21	4.50	6.09
	3	4.57	4.37	5.45
	4	4.49	4.73	6.36
	Avg.	4.35	4.55	5.76
Actual average increase.....		1.08	1.54	0.87
Increase.....		33%	51%	17%

the same set of twenty animals in each of the bottles 1, 2, 3 and 4 for the first and third periods will show that the increase is similar in all the bottles. For lot A it is 0.94, 0.91, 1.31 and 1.18 for bottles 1, 2, 3 and 4 respectively, while for lot B it is

1.86, 1.41, 1.50 and 1.37 for bottles 1, 2, 3 and 4 respectively. It might seem that the previous life in low oxygen did have an effect which resulted in slightly decreasing the acceleration in aërated water. This therefore, so far as it goes, confirms the inference from the comparison of the curves in Figs. 1 and 2 above in respect to the difference in the limiting concentrations of oxygen in the two methods of experiment used in obtaining the two curves.

A second question upon which the experiment given in Table III. was intended to throw light is: What effect does the state of nutrition have upon the magnitude of the increase of oxygen consumption during the third period? The animals of lot B were treated exactly like those in lot C except that those in lot C were fed liver about forty-two hours before the beginning of the first test period. The expected marked rise in oxygen consumption occurs in lot C during the first period. This rise persists during the third period but it will be noted that the increase in rate during the third period over that in the first period is only 0.87 c.c. per gram per 24 hours in the fed lot C as compared to 1.54 c.c. per gram per 24 hours in the starving lot B. The average increase in lot C is 17 per cent. while in lot B it is 51 per cent. This question was tested further by comparing fed and starved animals. Table IV. is self-explanatory. The animals in bottles numbered 5 to 8 were fed beef liver twenty-four hours previous to the experiment. The results again show a greater increase of oxygen consumption by the starved than by the fed animals, during the third period.

It was suspected that the increased rate of oxygen consumption during the third period might be due to increased motor activity by the animals during the third period. Accordingly in an experiment, the results of which are given in Table V., the heads of both starved and fed animals were cut off after feeding, that is, about 24 hours before the beginning of the experiment. All the data are calculated on the basis of the weights of the animals before feeding after subtracting the weight of the heads which were removed after feeding. The results on such decapitated fed and starved animals show the same relations in respect

TABLE IV.

All *Planaria* starved 26 days before experiment. Temp. 20° C. 7 days before and during experiment. 18 animals in each bottle.

		Period I. 6 hours.	Period II. 9 hours.	Period III. 6 hours.	
		O_2 Concentration in c.c. per 136 c.c. Water.			
		0.672	0.013	0.674	
	Bottle.	Total c.c. O_2 Consumed During the Period.			
Starved.....	1	0.083	0.005	0.119	
	2	0.100	0.005	0.122	
	3	0.100	0.005	0.116	
	4	0.126	0.010	0.136	
Fed.....	5	0.169	0.012	0.183	
	6	0.178	0.015	0.192	
	7	0.185	0.012	0.202	
	8	0.174	0.017	0.173	
		Average. C.c. Oxygen Consumed per Gram Weight per 24 Hours.			
		Period I.	Period II.	Period III.	Increase.
Starved.....		3.030	0.127	3.650	20%
Fed.....		5.220	0.204	5.540	6%

to the increased rate of oxygen consumption during the third period, as that found in the whole animals used in Tables III. and IV. The absolute increase during the third period, among the different experiments, is not accounted for by any difference in the length of the starvation periods of the animals previous to the experiments, for these were the same. On the other hand, any differences in motor activity which may have occurred in the different experiments do not seem to me to give any adequate physiological explanation of the effect. The magnitude of the effect of movement on the respiratory exchange may be inferred from certain observations on *Planaria agilis* made by Allen ('19, I.). There remains the alternative explanation, that the increase in oxygen consumption after living in low oxygen concentration is due to the oxidation of lower fatty acids or other by-products

TABLE V.

All *Planaria* starved 33 days before experiment. Temp. 20° C. 30 days before and during the tests. One half of the total number of worms were fed beef liver 24 hours before beginning of the tests. *Heads of all the worms were cut off one hour after feeding.* 20 worms in each bottle.

			Period I.	Period II.	Period III.	
			O_2 Concentration in c.c. per 136 c.c. water.			
			0.752	0.030	0.748	
	Bottle.	Weight of Headless Worms, gms.	Total c.c. O_2 Consumed During the Period.			
Fed.....	1	0.146	0.207	0.013	0.200	
	2	0.143	0.203	0.008	0.215	
	3	0.139	0.195	0.010	0.196	
	4	0.138	0.212	0.010	0.222	
Starved.....	5	0.132	0.106	0.005	0.142	
	6	0.133	0.125	0.008	0.116	
	7	0.131	0.111	0.002	0.135	
	8	0.135	0.115	0.007	0.127	
			Average, C.c. Oxygen Consumed per Gram Weight per 24 Hours.			
			Period I.	Period II.	Period III.	Increase.
	Fed.....		4.34	0.16	4.44	2%
	Starved.....		2.57	0.07	2.93	14%

of catabolism which accumulated during the period of life in low oxygen.[5] In the absence of a complete chemical study of the metabolism of *Planaria* the results await an adequate explanation.

[5] In his monograph "Respiratory Exchange of Animals and Man," Chapter VI., page 83, Krogh states that, according to Lesser (*Zeitschr. f. Biol.*, vol. 54, pp. 1–17), earthworms after living in the absence of oxygen, when returned to air show an increased rate of oxygen consumption, due to oxidation of accumulated oxidizable residues during the anaërobic period. In fact Lesser found just the opposite and states definitely, pp. 11–12: "Durch Respirationsversuche . . . wurde festgestellt, das der respiratorische Quotient beim Regenwurm . . . in der Erholung nach vorausgangener Anoxybiose erhöht ist gegenüber dem normalen. Es findet mithin in der Erholung eine völlige Verbrennung . . . der früher als Hauptprodukt der Anoxybiose gefundenen Fettsaüre nicht statt."

The Rate of CO_2 Production in the Absence of Free Oxygen.

The rate of carbon dioxide elimination by *Planaria* was determined by the use of a modification of the method described some time ago by Lund ('19). An important improvement in the method was brought about by washing the bottles with a stream of CO_2-free air, after the animals were placed in the bottles, thus doing away with the correction for CO_2 present in the air at the beginning. Where CO_2 production was determined in the absence of oxygen the bottles were washed out with hydrogen. All titrations were performed without removing the stopper from the bottle, by means of the simple expedient of boring two holes, one on each side of the bottle. These holes were fitted with stoppers and titration was carried on through one of these small openings. This procedure simplifies the method and increases its accuracy. The method has been used extensively in this laboratory and has proven itself to be satisfactory, especially when periods of two to three hours or more are used and comparative results are desired.

Table VI. gives the results of an experiment where twelve lots

Table VI.

Planaria starved and kept at 20° C. 38 days before experiment. Temperature during experiment 21° C. Twelve bottles with 50 animals in each. Blanks run as controls are not given in the table. 1 c.c. N/100 HCl equivalent to 0.111 c.c. CO_2.

	Duration of Test in Hours.	5	10	15
Air...	Total c.c. CO_2 produced per gram during test.	0.645 0.504	1.332 1.561	2.041 2.241
		Avg. 0.574	1.446	2.141
	Average c.c. CO_2 produced per gram during last 5 hours of test.....	0.57	0.87	0.69
H_2....	Total c.c. CO_2 produced per gram during test.	0.708 0.766	1.371 1.345	2.140 2.077
		Avg. 0.737	1.358	2.108
	Average c.c. CO_2 produced per gram during last 5 hours of test.....	0.73	0.62	0.75

of fifty animals in each lot were used. Six bottles were filled with CO_2-free air while another lot of six bottles were filled with hydrogen. The CO_2 produced by the worms in two bottles from each of the series filled with air and hydrogen respectively was determined at the end of 5, 10 and 15 hours, as shown in the table. The amounts of CO_2 produced during each one of the successive periods of five hours in air was 0.57 c.c., 0.87 c.c., 0.69 c.c. CO_2; and in hydrogen 0.73 c.c., 0.62 c.c. and 0.75 c.c. CO_2. The lots of fifty worms each were of course selected carefully as to size, history, etc., so as to render them comparable. The table shows clearly that no marked difference in the rate of CO_2 elimination occurs in the animals in air and those without free oxygen. All animals were normal at the end of the experiment.

Table VII. is a similar experiment of longer duration. The

TABLE VII.

Planaria starved and kept at 20° C. 11 days before the experiment. Temperature during the experiment 19.5° C. Twenty-five worms were placed in each bottle in 2 c.c. water. Blanks run as controls are not given in the table. 1 c.c. N/100 HCl equivalent to 0.111 c.c. CO_2.

	Duration of Test in Hours.	7	14	21	28
Air...	Total c.c. CO_2 produced per gram during test.	0.89 0.72	1.65 1.86	2.99 3.45	4.17 4.17
		0.80	1.75	3.22	4.17
	Average c.c. CO_2 produced per gram during the last 7 hours of test.....	0.80	0.95	1.47	0.95
H_2....	Total c.c. CO_2 produced per gram during test.	0.70 0.58	1.84 1.46	— 2.97	2.95 3.41
		0.64	1.70	2.97	3.18
	Average c.c. CO_2 produced per gram during the last 7 hours of test.....	0.64	1.06	1.27	0.21

carbon dioxide elimination during the successive four periods of seven hours each is in air 0.80 c.c., 0.95 c.c., 1.47 c.c., 0.95 c.c., while in hydrogen it is 0.64 c.c., 1.06 c.c., 1.27 c.c., 0.21 c.c. A comparison of the condition of the animals at the end of the 7-, 14- and 21-hour periods showed all the animals in hydrogen to have a normal response to light and touch. While at the end of twenty-

eight hours irritability was practically lost in those living in hydrogen. The animals in air were normal. The loss of irritability is clearly correlated to the fall in rate of CO_2 elimination during the last seven-hour period. All animals in this twenty-eight-hour period in hydrogen recovered when removed to air.

Another similar experiment which lasted thirty-eight hours gave similar results. A fall in rate of CO_2 elimination in hydrogen was again associated with loss of irritability and muscular tone. One set of animals in the thirty-eight-hour period only partially recovered when removed to air-saturated water. The controls in air were normal.

It is evident that the loss of irritability and appearance of a condition which closely resembles narcosis is correlated more closely to decrease in rate of CO_2 production than the lack of free oxygen.

An important fact to notice is that the action of KNC and the effect of absence of free oxygen are quite distinct. The experiments above show clearly that the rate of CO_2 elimination is not noticeably changed for hours after free oxygen has been removed. Potassium cyanide in proper concentrations inhibits oxygen consumption to as much as 70 to 80 per cent., as shown by Allen ('19, I.) and confirmed by Hyman ('19), and also markedly inhibits carbon dioxide elimination as found by Child ('19). While the absence of free oxygen does not affect the rate of CO_2 production for many hours, so far as the evidence goes, the return of *Planaria* to air-saturated water from KNC solutions strong enough to cause an inhibition of 50 to 80 per cent. does not result in an increase in the rate of oxygen consumption, after the return to air-saturated water, as is the case after life in the absence of free oxygen. Another fact to be noted is that it has not so far been possible to obtain a complete inhibition of the oxygen consumption in *Planaria agilis* by means of KNC. These differences in the action of KNC and lack of oxygen have not been fully recognized by previous investigators, while it has often been assumed that lack of oxygen and KNC solutions have the same effects.

Summary.

1. Oxygen concentration becomes a limiting factor in the rate of oxygen consumption by *Planaria agilis* at about one third air saturation of water at 20° C.

2. Life in low oxygen concentrations results in an accelerated respiratory metabolism varying from two to eighty-five per cent. when the animals are returned to air. This increase is not proportional to, nor directly dependent upon, the previous length of time which the animals have lived in what practically amounts to an absence of oxygen. Some slight evidence was obtained which indicated that repeated subjection of *Planaria agilis* to low oxygen concentration may result in a small decrease of the acceleration of oxygen consumption when returned to aërated water after living in very low oxygen concentration.

3. The percentage acceleration of oxygen consumption referred to under 2 is more marked in starved than in fed animals.

4. The rate of carbon dioxide elimination continues at practically the same rate in hydrogen as in air, until loss of irritability and muscular tone begins. The loss of irritability in *Planaria agilis* is therefore more closely correlated to change in rate of CO_2 production than to consumption of free oxygen if in fact it is correlated closely to either one.

5. The effect of KNC in inhibiting the oxidations in *Planaria* is not identical with the effects due to the absence of free oxygen.

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THE BEHAVIOR OF CELLS IN TISSUE CULTURES OF FUNDULUS HETEROCLITUS WITH SPECIAL REFERENCE TO THE ECTODERM.[1]

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INTRODUCTION.

Human, mammalian, avian and amphibian tissues have been frequently employed for the study of cells in tissue cultures but comparatively few observers (Osowski 1914, Lewis 1916 and Dobrowolsky 1916) have used fish tissue. These embryos are easily obtained, the culture media are simple to prepare, and large growth takes place at room temperature. They therefore constitute an ideal material in which to study the movement of the epithelial membrane, the structure of the ectoderm cells and their relation to other cells.

TECHNIQUE.

Pieces of *Fundulus* embryos were explanted into diluted sea water in the manner described by M. R. Lewis ('16). As an additional precaution against infection, the eggs were dropped into 95 per cent. alcohol for one second and then transferred to sterile sea water. In preparing the media several dilutions of sea water were employed: *i.e.*, 20 per cent., 30 per cent., 35 per cent., 40 per cent. and 50 per cent. in distilled water. To 80 c.c. of each of these dilutions was added 20 c.c. *Fundulus* bouillon, 0.02 gm. $NaHCO_3$, and 0.5 gm. dextrose. The media were then sterilized.

Growth was obtained in dilutions of sea water ranging from 20 to 50 per cent., although in the latter it was infrequent and not of great extent. While growth was satisfactory in a dilution of 20 per cent., the proportion of good growths was larger with 30 per cent. and still larger with 40 per cent. sea water. More-

[1] From the Marine Biological Laboratory, Woods Hole, Mass.

over, cell proliferation from each piece was more abundant. Cultures grew in media containing no dextrose, or with varying amounts of dextrose up to 2 per cent. Media containing 0.5 per cent. gave the best results. The cultures grew equally well with 80 per cent. of Locke's solution in place of sea water. Good results were obtained with 80 per cent. of Locke's solution and 20 per cent. of chicken bouillon substituted for the fish bouillon, although not so many cultures grew. Pure egg albumen and dilutions of this added to the normal medium likewise proved successful.

Several cultures which contained tissue from both chick and *Fundulus* embryos were made in Locke's solution, chicken bouillon and dextrose. These were kept in the incubator at 39° C. and at the end of 48 hours the explants showed equally good outgrowths.

Cultures were usually prepared in the afternoon, and by the following morning growths in the form of membranes had formed, though sometimes this did not occur until the second day, especially when a dilution of 20 per cent. sea water was used. The embryos were 7, 10, 14 and 15 days old (just before hatching) but the age made no apparent difference in the growth from the explant.

General Characteristics of the Cultures.

Examination of the cultures shortly after explantation usually showed a few isolated mesenchyme cells, which soon began to migrate outward on the cover-slip. The ectoderm cells in the region of the cut edges rounded up into more or less spherical bodies. After several hours typical cultures revealed a membranous outgrowth of ectoderm from one or more regions of the explant, and beyond this an area of mesenchyme cells, isolated, or forming a reticulum upon the under surface of the cover-glass (Fig. 1). Nerve fibers, projecting freely into the fluid along the coverglass, were often present in abundance. Pigment cells and yolk-filled cells from the digestive tract (Fig. 7) were also common. No outgrowth of muscle cells was observed.

A renewal of the fluid medium was not attempted in any of the cultures. The oldest healthy cultures were ten days old,

and in one of these the heart was still beating at the time of fixation. In one culture peristalsis was observed in a portion of the intestine which projected out into the medium. Contractions of the trunk musculature, beating of the heart, and movements of the fins were frequently observed in cultures several days old.

Behavior of Ectoderm Cells at Cut Edges.

In normal embryos the ectoderm consists of a single layer of large pavement cells, polygonal in surface view, which may be clearly seen in the regions covering the fins and the trunk musculature. The cells are transparent and almost colorless. The internal structures are only vaguely visible. The cells covering the trunk have delicate concentric markings, suggesting somewhat the markings upon fish scales.

When the embryos are cut the ectoderm cells along the edge round up very markedly into spherical masses, especially in the region of the heart and the yolk sac. Any small group of cells or single cells that have in cutting become separated from the explant remain for some time in this state. Eventually, large vacuoles develop in them and they remain inactive in this condition for several days before disintegration takes place. Similar cells were frequently observed in old cultures along the edge of the ectodermal membrane.

The direction of the cut is a factor in the successful growth of cultures from the trunk region of the body. The cells do not grow out unless the cut is irregular or oblique. If it is transverse the cells close in and form a covering over the injured end, preventing outward migration. Osowski found that injured surfaces of fish embryos were covered by an epithelial membrane within twenty-four hours.

Membranes never grow out from cut fin surfaces; the cells near the cut surface become wrinkled and irregular in contour, and remain in this condition sometimes for several days, before they become rounded up and display the large vacuoles and greenish protoplasm which are characteristic of degenerating ectoderm cells. Sometimes the ectoderm of the fins degenerates

even when not cut, although the other cells of the culture exhibit normal activity.

Ectodermal Membrane.—Fig. 1 shows the extent of a characteristic membrane in a seven-day culture. In the living cultures the ectodermal cells spread out in a very thin, flat and colorless layer on the under side of the cover-glass. The position of the nucleus and of the granules within the cell could rarely be detected. At the edge of the membrane the cells were thicker and darker in appearance, with a greenish tinge and of very irregular form. Projecting beyond the ectoderm, mesenchyme cells could be seen adhering closely to the cover-glass. Other cells, slightly darker and containing vacuoles and granules, were observed migrating above the ectodermal layer. Slides stained with iron hæmatoxylin show that such outgrowths from the explant consist of a practically continuous ectodermal membrane of extremely flat, slightly granular cells, and a more or less imperfect membrane of mesenchyme lying above it, closely adhering to the cover-glass (Fig. 2). The mesenchymal membrane is never so perfect as the ectodermal layer, and there is a gradual transition from a membranous form to more or less isolated cells which may project for a considerable distance beyond the ectoderm (Fig. 3). One group of ectodermal cells was found which was not covered on its upper surface by mesenchyme (Fig. 8). These cells were characterized by very granular nuclei containing one or more nucleoli, and by mitochondria in the form of threads and granules. Frequently the cells showed a rosette-like arrangement around a small intercellular space. Mesenchyme cells were readily distinguishable in the stained slides by their more granular cytoplasm and indefinite cell boundaries. The size of the nuclei was not a criterion, as the nuclei might be either larger or smaller than those of the ectoderm. Mitochondria were much more abundant than in the ectoderm and stained more deeply. Fig. 9 shows a group of mesenchyme cells, unaccompanied by ectoderm, in which the mitochondria appear very clearly. They were also observed in the living cells when stained with janus green. Certain other granules became visible when stained with neutral red. The latter stain also affected granules in the thickened

cells at the edge of the ectoderm, but the presence of such granules in the flat ectodermal cells could not be determined with certainty. These cells appeared less easily penetrable to a number of vital dyes, while the mesenchyme cells were readily colored.

Formation of Ectodermal Membrane.—In a study of fetal skin growing in blood serum, Loeb ('12) observed that the ectoderm cells migrated into the surrounding medium in the form of strands. Holmes ('13) and Uhlenhuth ('14) described a similar condition in the frog. In my observations on fundulus the ectodermal outgrowths were invariably in the form of very thin, one-layered membranes. The ectoderm never migrated in strands. The earliest appearance of the membrane was indicated by an exceedingly thin, flat layer near the explant which was continuous with the rounded cells covering the body region, and bordered along its outer edge by a broad mass of irregular thickened cells. The mesenchyme cells lay in a thin sheet above the ectoderm and projected beyond it. As the rounded cells migrated from the explant their contour changed gradually, and they became flattened with irregular, thickened, central portions which projected downward in the fluid medium. Text-figure 1 is a drawing of an ectodermal cell in the process of flattening out during its migration. The portion of the cytoplasm spread out along the cover-glass formed a clear thin area bounded by a cell wall which was in close contact with the walls of neighboring cells. In this clear region were a few pale concentric markings. The central mass of thickened cytoplasm eventually disappeared as the cell flattened out completely. A few hours later, as a result of the migration and flattening of the cells, the membrane had increased more than twice in extent, and no thickened cells remained except a few scattered ones and a single row of elongated cells around the edge.

Changes at the Edge of the Ectodermal Membrane.—The edge of the ectodermal membrane was seen to undergo slow and continual changes during the active growth of the culture. Usually the cells were thickened and elongated or extremely irregular in form, with numerous blunt knobs projecting downward. Their cytoplasm appeared granular in contrast to the

apparently homogeneous protoplasm of the flat cells. They were observed to flatten out and thus extend the membrane. It seemed as though the cells were under unequal tension for as the membrane grew wrinkles formed in cells that had formerly been flat. The wrinkles could be differentiated from the cell thickenings previously described, for they were obviously folds in the membrane and often involved more than one cell.

Ectodermal pseudopodia, formed from a hyaline outer region of the cells, have been observed by Harrison ('10) and by Holmes ('13) in tissue cultures of the frog. In the ectoderm of fundulus, however, the entire cell is equally hyaline when stretched out flat, and the formation of pseudopodia was never observed.

The ectodermal membrane is not only very extensible, but elastic as well. In staining the cultures with vital dyes the greatest care was necessary to prevent its retraction, which often followed within a few seconds. This is also likely to occur if the slides are jarred. In one culture the ectoderm cells along the edge contracted and thickened, pulling along with them large portions of the mesenchyme membrane. This double membrane then rolled in upon itself and, as it tore loose from the cover-glass, could be seen adhering by short projections from certain of the cells. These in turn loosened and the membrane pulled in farther (Fig. 5). In such cases of mechanical disturbance of the culture the membrane later became reduced by the contraction of the ectoderm to a compact mass of cells which eventually disintegrated. Ruth ('11) describes the contraction of the edges of the growing epithelial cells during the healing of a wound in the skin of a frog in vitro.

Striations in Ectoderm Cells.—The most peculiar characteristic of the ectoderm cells is the presence of numerous delicate striations, more or less concentrically arranged, which form an intricate pattern over all the cell (Figs. 3. and 10). The markings did not appear on all of the cells and only occasionally were they sufficiently clear in the living cultures to be drawn with the camera lucida. When the cells began to spread out and migrate from the cut surface of the embryo, a few markings could be seen in the flat clear portion of the cell (text-fig. 1) and, as this region

increased in extent, more striations became visible. The markings appeared to be on the under surface of the cells, that is, farthest from the mesenchyme. The striæ were very definite, appearing as longer or shorter dark lines over the cell, varying only slightly in width, and never crossing a cell boundary. The wall between adjacent cells was distinctly double, so that each cell had its own complete investment. This was also clearly seen in a few cases where ectoderm cells had become separated from each other. Frequently one or more striæ near the periphery of a cell were situated parallel with the cell wall, while the inner ones were more irregular or arranged concentrically with reference to several points in the cell.

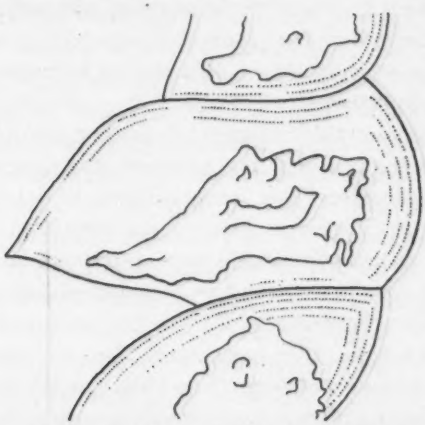

FIG. 1. Camera lucida drawing of an ectodermal cell in process of flattening out during migration; the central mass of thickened cytoplasm bordered by a clear flat region with pale concentric markings. Ocular 5, lens 4 mm.

Form and Behavior of Mesenchyme Cells.—The mesenchyme cells grew out from the explant as more or less elongated and separate cells, which later became connected in various ways. Adjacent cells sometimes sent out short broad processes along their sides, which appeared to fuse with each other, forming a reticulum with relatively small intercellular spaces (Fig. 9). Characteristic also of these cells was the projection of their protoplasm into short thread-like processes often giving a prickly appearance to the edge. The prickly processes became converted

into protoplasmic bridges connecting the cells. When these were present the tissue appeared somewhat similar to the mesothelial membrane described by Lewis and Lewis ('12) in tissue cultures of the chick, except that in fundulus the cell boundaries are much less distinct. Whether the cells actually fuse or not is a question which is extremely difficult to determine with certainty. When the cells are connected with each other by extremely elongated protoplasmic processes, as shown in Fig. 3, the intercellular spaces are large and a wide-meshed reticulum results. There is a gradual transition from a membranous to a reticular arrangement of cells as the distance from the explant increases.

The outward growth of these reticular cells appears to be an important factor in the extension of the ectoderm. As previously noted, reticular mesenchyme cells were usually seen projecting beyond the ectodermal membrane (Figs. 1 and 3). These cells underwent very marked changes in form, their protoplasm flowing in a direction away from the ectoderm, the proximal portions of the cells, however, being connected directly or by intervening cells with the thickened edge of the ectoderm. The distal portions of the cells frequently developed broad fan-like expansions, which were firmly anchored to the cover glass.

The mode of formation of the fan-like expansions appears to be about as follows: The pseudopodia flow out from the cell in the form of delicate, radiating, thread-like or finger-like processes; then the region between the processes gradually fills out until a fan-like form is attained. This soon becomes entirely homogeneous near the periphery and adheres closely to the cover glass. Frequently the fan has a slightly fluted appearance as if not adhering equally well at all points.

As the mesenchyme cells migrate outward they exert a pull upon the edge of the ectoderm which is drawn outward, not by amœboid processes of its own, but by amœboid processes and fan-like expansions of the mesenchyme cells to which it is attached. The sheet of ectoderm is thus anchored in all directions as if by minute guy-ropes. When these are pulled more in one direction, folds and wrinkles are formed, which become smoothed out when the pull is equalized.

Pigment Cells.—Chromatophores of fundulus are large cells containing either black, brownish red, or yellow pigment granules. The cells migrate readily from the explant, but do not as a rule travel far. Stockard ('15) showed that the chromatophores have an affinity for plasma-filled spaces, being found adhering to the pericardium in normal embryos and to the heart itself in embryos deprived of a circulation. In the cultures of fundulus brown chromatophores were several times observed closely adhering to the beating heart, which happened to be projecting out into the fluid medium. Here they remained elongated for several days until the cultures degenerated. Isolated pigment cells that had wandered out upon the membrane degenerated more rapidly than other kinds of cells, and the pigment granules, freed from the cells, were readily taken up by the mesenchymal cells where they became aggregated around the nucleus (Fig. 6).

In addition to the pigment cells and those forming a membrane or reticulum other types of mesenchyme cells were observed. Among these were certain denser, more granular and vacuolated cells which migrated out upon the mesenchymal reticulum, and other cells (probably clasmatocytes) with brighter, more solid-looking protoplasm and numerous curved, finger-like pseudopodia. These cells contained numerous granules often derived from degenerated chromatophores. Brownian movement of these ingested granules was frequently observed.

Yolk Cells.—In some explants certain peculiar spherical cells filled with numerous clear greenish yolk spheres were seen massed together in the anterior part of the digestive cavity. These cells migrated readily out upon the mesenchymal reticulum where they became very slowly amœboid and wandered out along the edge of the ectoderm. Fig. 7 shows a group of these cells with the yolk spheres stained deep black with iron hæmatoxylin.

Cell Division.—The appearance of new cell boundaries in the ectoderm was frequently observed but in no case was a cell seen to divide. There is therefore no evidence as to whether the cells divide by mitosis or amitosis. In over fifty cultures stained with iron hæmatoxylin no stages of mitosis were observed. Two

nuclei, however, were common in ectoderm cells (Fig. 8), as were also nuclei partly constricted into two, or with several irregular constrictions and variable nucleoli. There is no reason to believe that this condition is followed by division of the cells. It may be that the nuclei fuse together again, as Macklin ('16) observed in tissue cultures of the chick, and that the cells subsequently divide by mitosis. Holmes ('13) observed amitosis of the nuclei in tissue cultures of various tadpoles, but he states that nuclear division was not followed by division of the cytoplasm.

A few mesenchyme cells were observed to divide by mitosis and several groups of chromosomes in metaphase appeared in the stained material. Bi-lobed and double nuclei, indicative of amitosis, were also observed (Fig. 9).

Cultures of Chick and Fundulus.—A piece of fundulus tissue and a piece of muscle tissue from a chick embryo of eight days' incubation were placed together in a drop of Locke's solution containing chicken bouillon, and kept at 39° C. At the end of 48 hours each piece showed its characteristic form of growth, the fundulus having the double membrane previously described, the chick tissue showing the usual radiating type of outgrowth. In one region the fundulus outgrowth could be seen growing over a portion of the chick explant as over a foreign body. In another region outgrowths from the two pieces were almost in contact, but the cells from the two explants showed no tendency to intermingle. Specific differences were observable within the cells. Mitochondria are much more abundant in the chick tissues and the cytoplasm appears to be different, as shown by the greater ease with which the cellular structures of the chick may be observed.

General Considerations on the Movements of Membrane Cells.—The behavior of the cells in the cultures at different times is of considerable interest. Taking the normal form of the ectoderm cells as a standard, we find that the cohesive property of these cells is increased suddenly at the time the cut is made, as shown by the rounding up of the cells. The stimulus of the injury was sometimes effective for several hours. This influence seemed to

be gradually weakened and the adhesive power progressively increased as the cells spread out in a thin layer upon the under surface of the mesenchyme. Holmes ('13) found that ectoderm cells of tadpoles in tissue cultures attach themselves readily to various kinds of substrata, including the cover glass, and "extend upon one another in mutual attraction which tends to keep them in continuous masses." In fundulus, however, this stereotropic activity of the ectoderm is called forth only when the cells are associated with the mesenchyme, and it appears to be much stronger than in the frog, causing the cells to be spread out in a thin single-layered membrane. In over fifty cultures it was never observed that an ectodermal membrane grew out unaccompanied by mesenchyme, whereas numerous cultures contained growths consisting of mesenchyme alone. The latter cells appear to be more highly stereotropic than the ectoderm, for they will adhere to the smooth surface of the cover glass even to the extent of having their processes snapped off when the ectoderm retracts.

The difference in behavior of the two layers of cells is perhaps correlated with the fact that under normal conditions of development ectoderm cells grow only in contact with the mesenchyme, whereas mesenchyme cells can grow in contact with widely varying kinds of surfaces.

The question arises whether there is any relation between wound-healing and the formation of the ectodermal membrane in tissue cultures. Loeb ('20) has discussed various processes involved in cell movements in wound-healing, designating among others amœboid migration of ectoderm cells, this being "the first response of the tissue to the wound stimulus." A factor in this amœboid wandering of the ectoderm cells is their stereotropic reaction, as expressed by their contact with the coagulum, which is "the foundation for the process of wound healing." In fundulus cultures the wandering of the cells in contact not with a coagulum but in this case with the mesenchyme layer is the foundation for the process of formation of the ectodermal membrane. The manner of cell movement, however, does not appear to be amœboid in character.

Oppel ('13) has described the bending of the skin edges along the cut surface of explanted pieces of the tadpole's tail as due to real movement; not simply a mechanical process but a change in form by which the ectoderm grows around the cut. In an earlier paper ('12*a*) he compares the ectoderm cells to partly filled sacs of inelastic material which can change their outline without varying the extent of their surface. This kind of movement is strikingly similar to the early changes which take place when the rounded cells begin to flatten. Oppel ('12*b*) distinguishes between epithelial movement, which is a mass movement, and amœboid motion which tends to isolate cells, as in connective tissue. He concludes that while the movement of the epithelium depends on the activities of the cells themselves, it is not an amœboid motion. The observations on fundulus confirm this interpretation. Here epithelial movement appears to be a mass movement throughout all stages in the formation of the ectodermal membrane. Single functional epithelial cells are never found.

In conclusion it may be stated that the activities of the cells in the formation of the ectodermal membrane in fundulus are similar to activities displayed also by ectoderm cells in the process of wound-healing. As in the latter, the cells exhibit mass movements the end result of which is to cover the connective tissue; so in tissue cultures of fundulus the migration of the ectoderm cells proceeds by mass movement which results in a partial covering of the mesenchyme layer. The contact reaction toward underlying connective tissue, exhibited by ectoderm cells in wound-healing, is paralleled by the stereotropic activity of the ectoderm cells evoked by contact with the mesenchyme. It may be said that the cells are attempting to follow out their normal activities, although subjected to abnormal conditions.

Summary.

1. Tissues of *Fundulus heteroclitus* grew in fluid media under conditions varying widely in respect to temperature, concentration of salts, and character of nutritive substances.

2. Mesenchyme cells migrated out into the medium upon the under surface of the cover-glass and formed almost continuous

or reticular membranes with isolated cells lying beyond. The ectoderm formed a membrane in close contact with the under surface of the mesenchyme. Nerve fibers, pigment cells, and yolk cells from the digestive tract readily migrated out. Peristalsis of the intestine, beating of the heart, contraction of the trunk musculature, and movements of the fins, were observed in numerous cultures several days old.

3. Characteristic of the ectoderm cells were certain delicate striations somewhat concentrically arranged, which formed an intricate pattern over the cell.

4. The mesenchyme cells are highly amœboid and possess characteristic fan-like expansions by means of which they adhere to the cover-glass and to each other.

5. While the initial stages in the formation of the ectodermal membrane were accomplished by migration and flattening out upon the under surface of the mesenchyme of the cells originally covering the body, the further extension of the membrane involved the formation and growth of new cells and tension exerted by the mesenchyme upon the thick edge of the ectoderm.

6. Mitosis was observed in several mesenchyme cells but not in the ectoderm, although new cell boundaries appeared from time to time. Frequently ectoderm cells contained two nuclei or one irregularly lobed nucleus.

7. During all stages in the formation of the ectodermal membrane the movement of the cells is a mass movement. Their reactions are much slower than those of the mesenchyme, and are never amœboid in character.

8. There is an essential similarity in the outgrowth of the ectodermal membrane and the process of wound-healing in respect to (*a*) the mass migration of the ectoderm cells, and (*b*) the stereotropic activity of the cells which is evoked by contact with the mesenchyme.

I wish to acknowledge my indebtedness, for the valuable suggestions and criticism, to Prof. W. H. Lewis and Mrs. Lewis, of the Department of Embryology of the Carnegie Institution of Washington, under whose direction this work was accomplished.

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DESCRIPTION OF PLATES.

Figs. 1–10 are from photographs of cultures fixed in Zenker's solution without acid, and stained in iron hematoxylin. All figures except 1 and 5 were made with the high power. 1 and 5 were photographed with No. 4 Oc. and No. 16 lens.

PLATE I.

FIG. 1. Seven-day culture from 15-day embryo, showing extent of double membrane composed of ectoderm and mesenchyme. The latter projects beyond the thickened edge of the ectoderm in the form of a loose reticulum or of isolated cells.

FIG. 2. Portion of outgrowth from an explant showing a continuous ectodermal membrane of large flat cells, and an imperfect membrane of darker granular mesenchyme cells, which lies above it.

FIG. 3. Portion of a reticulum of elongated mesenchyme cells, attached at one end to the thickened edge of the ectoderm, anchored at the other to the cover-glass by broad fan-shaped expansions. In some of the ectoderm cells delicate striations are visible.

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PLATE II.

FIG. 4. Portion of edge of ectodermal membrane, the cells almost completely flattened and slightly curled back at their extreme edge; mesenchyme cells projecting beyond them.

FIG. 5. Result of contraction of the ectoderm due to mechanical disturbance. The dark mass at the left is a retracted membrane of ectoderm and mesenchyme. Other portions of the mesenchyme remained adhering firmly to the cover-glass.

FIG. 6. Group of ectoderm cells, with large nuclei and faintly granular cytoplasm. Nuclei of mesenchyme cells are smaller, and are surrounded by dark granules derived from degenerated pigment cells; the boundaries of mesenchyme cells are not visible.

FIG. 7. Group of yolk cells lying upon the mesenchymal membrane where they had migrated from the digestive tract. The yolk spheres are stained deep black with hematoxylin.

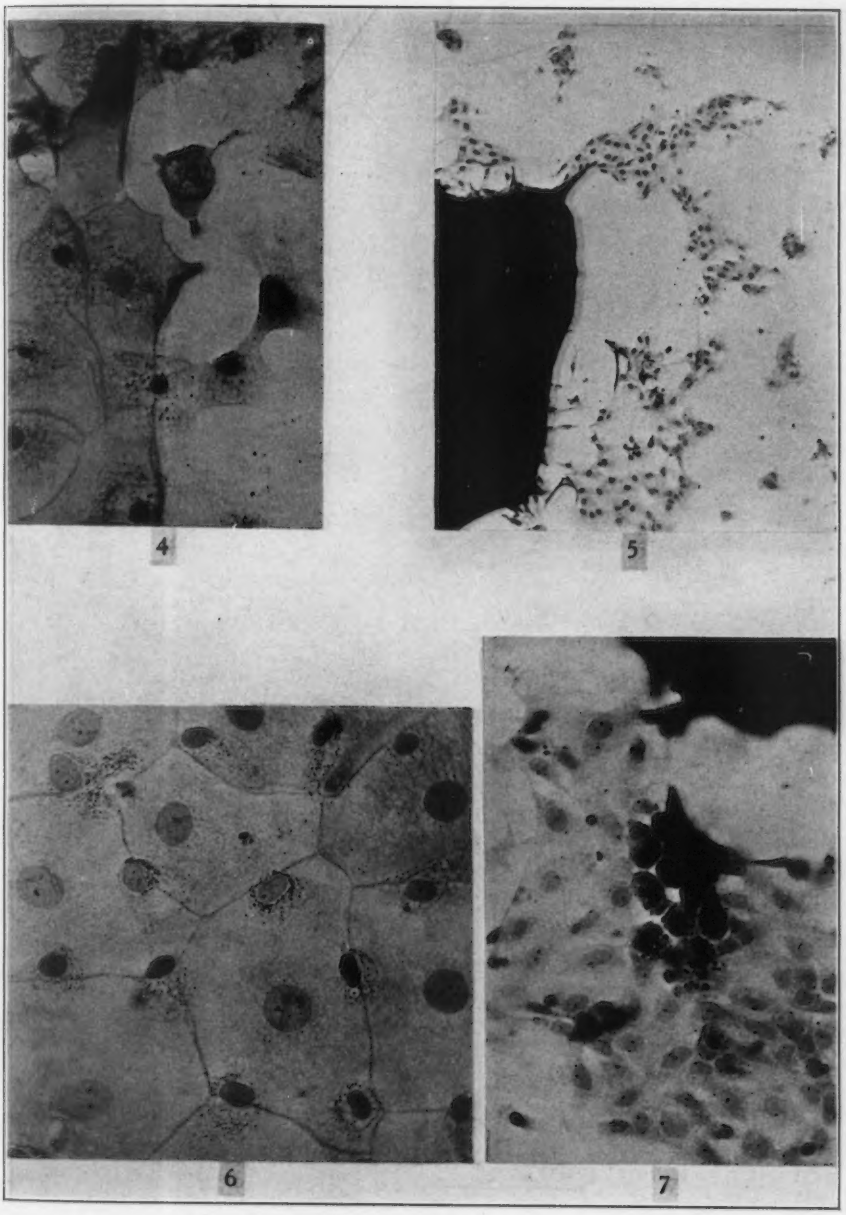

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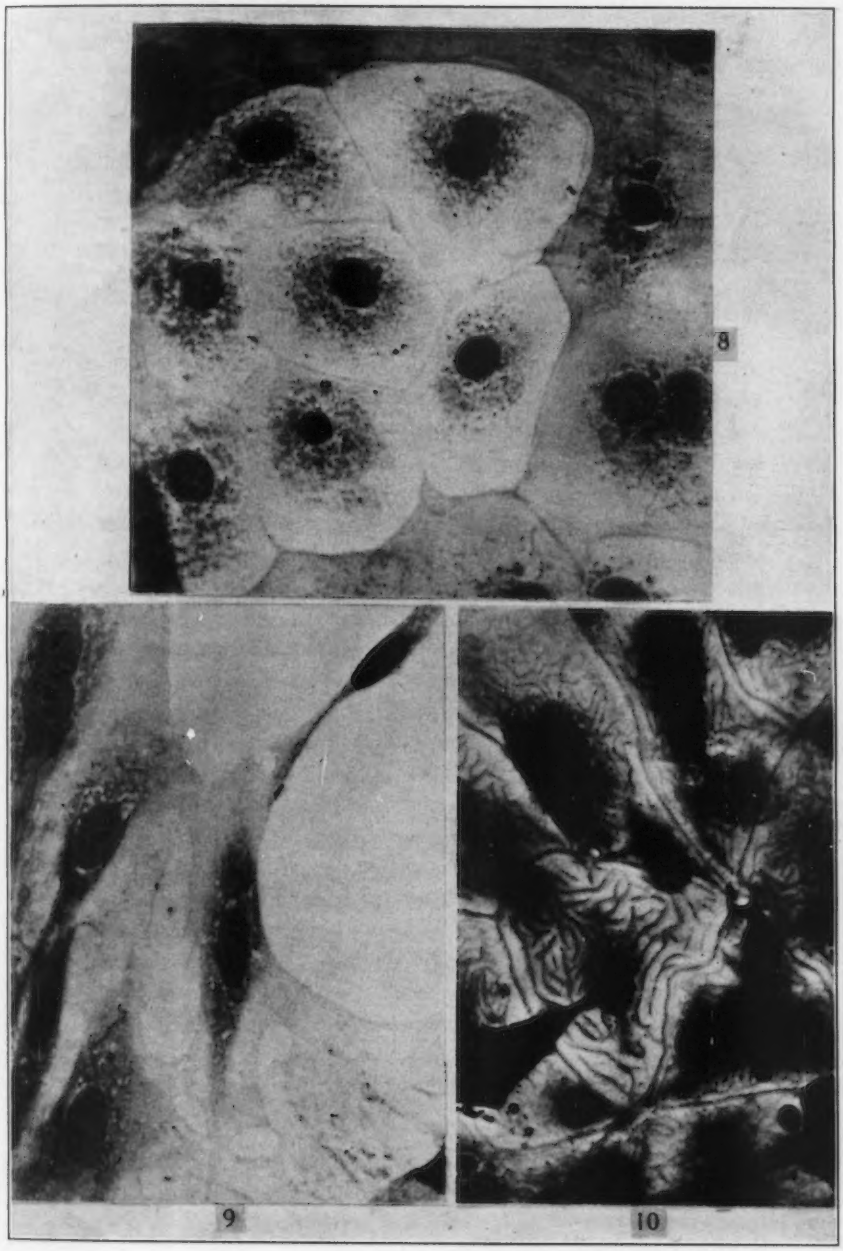
PLATE III.

FIG. 8. Group of seven ectoderm cells, free from overlying mesenchyme. A bilobed nucleus is visible in one cell.

FIG. 9. Group of mesenchyme cells forming a membrane with relatively small intercellular spaces. Mitochondria are visible in the form of threads and granules.

FIG. 10. Portion of outgrowth showing striations in ectoderm cells. Nuclei and cytoplasm of mesenchyme cells are visible as dark masses upon the ectoderm.



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THE PRESENCE OF GLYCOGEN IN THE CELLS OF EMBRYOS OF FUNDULUS HETEROCLITUS STUDIED IN TISSUE CULTURES.

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At the present time it is practically impossible to demonstrate the various chemical substances of the living cell, owing to the fact that most means of analysis cause the death of the cell. A few of the differential stains, such as Sudan III. and Nile blue, have been applied to studies of living tissues, but these do not behave in the same manner in the living cell as in dead material (Lewis and Lewis, 1915; M. R. Lewis, 1918). In most cases the chemical nature of cytoplasm has been discussed from the standpoint of results obtained from dead cells, and it is doubtful whether such conclusions can be applied directly to the living. This is true in regard to the experiments given herein; for, while the substance described reacts as does glycogen to the tests usually employed to demonstrate glycogen, these results were not obtained until after the cells had begun to be affected by the iodine.

Bernard (1859) demonstrated the presence of glycogen in many kinds of tissues and since that time several methods for showing this substance in the cell have been reported. The methods most frequently used for histological purposes are those of Best (1909), Gage (1917). Neither of these was used in the experiments given below. Instead, the living cells were exposed to iodine vapor in such a manner that they could be followed throughout the experiment, *i.e.*, when living, while dying, and after death had occurred.

TECHNIQUE.

Small pieces of fundulus embryos (just before hatching) were explanted into hanging drops of the following solution: 80 c.c.

diluted (40%) sea water plus 0.02 per cent. $NaHCO_3$ plus 20 c.c. fundulus bouillon plus 0.5 per cent. dextrose (M. R. Lewis, 1917). Within 24 to 48 hours large growths were present around these explants. The cells composing the growths were then exposed to iodine vapor by scattering a few fragments of an iodine crystal in the bottom of the hollow ground slide under the hanging drop, care being taken to prevent them from touching the drop. The vapor from the iodine crystal penetrated the hanging drop and acted upon the cells. Just a sufficient amount of iodine should be used to rapidly color the cytoplasm yellow and to show the port-wine color of the glycogen within one to two minutes.

Normal Fundulus Cultures.

As has been shown by Dederer (1921), the ectoderm and the mesenchyme cells grow out from explants of fundulus embryos in the form of membranes. The mesenchyme cells are usually attached to the cover-slip and the ectoderm cells form a layer directly beneath them. Mesenchyme cells extend beyond the ectoderm and also along the edge of the membrane and those scattered farther out on the cover-slip are the only cells which have processes to any extent. These processes usually spread out at one end into a large, thin, fan-like structure. One or more large, flat, oval cells, probably endoderm, are sometimes found on the membranes; these are quite different from the mesenchyme cells, in that they have an oval shape while the mesenchyme cells are elongated or somewhat hexagonal, and their cytoplasm also appears to be of a different consistency. For convenience of description these cells will be called *oval* cells. When the cultures were placed over fragments of an iodine crystal the cytoplasm and nucleus of all the cells became yellow, the the mitochondria a darker yellow, and the fat globules a brownish color. Almost the entire cytoplasm of the oval cells, regions of the cytoplasm of some of the mesenchyme cells, and certain parts of a number of their processes turned a port-wine color. This was a distinctly different tint from that exhibited by any other part of the growth and was the same as the characteristic color exhibited by glycogen when exposed to the action of iodine.

The three regions which showed this color reaction will be discussed separately.

1. *Oval Cells.*—As the iodine vapor penetrated the hanging drop, the cytoplasm of these cells became a pale yellow; then the granular portion, including the mitochondria and fat droplets, *i.e.*, the endoplasm, appeared to shrink slightly and became a deeper yellow. Meanwhile the remainder of the cytoplasm, except a thin yellow ectosarc, turned pink, the color gradually deepening until, after a few seconds, this portion was a deep port-wine color, while the endoplasm, nucleus and ectosarc remained yellow. At times the port-wine-colored material occupied the greater part of the cell, leaving only a small clump of endoplasm and the nucleus. In other cases the yellow endoplasm, nucleus and ectoplasm took up practically the entire cell except what appeared as a large port-wine-colored vacuole. The contour of the cell did not change but remained the same size as it was before exposure to the iodine. In a few of these cells there were a number of vacuoles, which also became port-wine color. The color was more intense in tone in the oval cells than in any other portion of the growth. It remained in them for some time, in a few instances for over an hour, then faded, and the whole preparation became a dark yellow color.

2. *Mesenchyme Cells of the Membrane.*—Of the cells forming the membrane probably only the mesenchyme exhibited the port-wine color upon exposure to iodine. This was difficult to determine definitely because the cells adhered together so closely, and also because of the fact that, while the mesenchyme cells were frequently found extended beyond the ectoderm cells, the latter were never observed separated from the former. Not all of the mesenchyme cells showed the port-wine color when in the presence of iodine, and which ones would do so could not be foretold. In these cells the cytoplasm of the central and thicker portion of the cell became a diffuse pink color, while the nucleus and ectoplasm became yellow. This area of pink coloration was not definitely limited but toned off into an extensive yellow ectoplasm. The arrangement of the mitochondria and other structures was the same in the cells having a pink area as in

normal cells. Gradually the pink color became deeper in tone until it attained a pale port-wine color. Within a few seconds the color disappeared from the central region of the cell and a large, round port-wine-colored bleb appeared at one side of the cell. Practically no change was observed in the structure of the cell during the disappearance of the stain and the formation of the bleb. Many of these blebs appeared scattered over the membrane, especially in the region adjoining the explanted piece. The color remained in them for about twenty minutes and then faded out, leaving the blebs rather undefined and difficult to distinguish. In some of the more peripheral cells of the membrane the port-wine color remained diffuse in the cytoplasm of the central portion for some time and then faded out without forming blebs. The mesenchyme cells which had migrated out on the cover-slip, away from the membrane, seldom formed blebs.

3. *Cell Processes.*—The processes of the cells formed the region where the appearance of the port-wine color could be observed most clearly. The large fan-like processes referred to above exhibited lighter and darker areas where the cytoplasm varied either in density or in thickness. After exposure to iodine some of the lighter regions became pink. Later these turned into distinct port-wine-colored areas in the yellow cytoplasm. A few of the processes did not exhibit these stained areas at all; in others some of the areas remained quite pale in color. This phenomenon did not continue for longer than half an hour; at the end of this time the color had faded and that area of the process was slightly shrunken.

When a drop of saliva was placed upon a culture which was later exposed to iodine vapor the port-wine color was not found in the cells. Death of the cell also prevented its appearance. No granules having the characteristic glycogen color were seen in any of the cultures. Neither the mitochondria nor any other granules were concerned in the formation of the port-wine-colored areas. It seemed as though the material which exhibited the typical port-wine color was diffuse throughout certain parts of the cytoplasm and became more definitely localized during the death of the cell, which occurred coincidentally with the iodine

staining. Thus it is seen that some substance, which reacts as does glycogen when exposed to iodine, is present in the cells of fundulus cultures. It is possible that this substance is glycogen. That the substance which became port-wine colored was not the dextrose itself is shown by the fact that dextrose placed in Locke's solution did not so stain when exposed to iodine, while glycogen did.

A few cultures of chick embryos were tested in the same manner for a comparison with those of the fish embryos. No port-wine color was observed in the cells of the older embryos except in one somewhat degenerate culture where a few blebs were already present on certain of the dying cells; these blebs became slightly pink but in no case was there the port-wine coloration such as occurs in fundulus cultures. On the other hand the cells in cultures of very young embryos (48 hours) sometimes contained an abundance of this substance.

The Influence of Starch Upon the Amount of Glycogen Present in the Cells.

Soluble starch (Kaulbaum) was added to the medium of the cultures of fundulus embryos in order to determine whether it could be utilized by the cells to store up glycogen. The starch was dissolved in distilled water and boiled for two minutes; 60 c.c. of the starch solution was then added to 40 c.c. of sea water and the medium prepared in the same manner as for normal cultures. When the quantity of starch was less than 0.1 per cent. it had no appreciable effect upon the cells. In these cultures the growth was normal and no increase in the amount of glycogen could be detected. When larger amounts of starch were added, or when the starch became slightly clumped into masses of very small granules, as sometimes happened, small particles were occasionally taken up by the cell and appeared within the cytoplasm as small granules or granular masses, in some cases surrounded by a vacuole. Upon exposure to iodine the starch became blue, whether within the cell or in the medium. The surrounding vacuole became pale blue, or sometimes lilac, but never the port-wine color indicative of glycogen. The port-

wine color was present in the same regions as in normal cultures, but never greater in amount and sometimes less than in the normal control preparations. Even after a number of days the starch did not become changed into glycogen as, for instance, one 8-day-old culture in 0.75 per cent. starch solution, exposed to iodine, exhibited cells in which there were a few vacuoles some of which contained blue granules. The vacuoles were pale blue or lilac but never port-wine color.

Influence of Dextrose in the Medium Upon the Amount of Glycogen in the Cells.

Cultures were prepared in a medium free from dextrose in order to ascertain whether the lack of dextrose would prevent the appearance of glycogen in the cytoplasm of the cells. The results from twenty such cultures show that, while the amount of glycogen could be decreased by the lack of dextrose in the medium, its presence could not be entirely inhibited. Some of these cultures (48 to 72 hours) exhibited only a slight trace, if any, of the port-wine color when exposed to iodine. On the other hand, a few did contain decided evidences of a small quantity of this substance.

Explants into media containing 0.5 per cent., 1 per cent. and 2 per cent. dextrose, made at the same time as those without dextrose, showed a decided increase in the quantity of glycogen up to a certain point. In no instance did all of the cells of a culture exhibit the port-wine color. Neither did any one cell become greatly filled with this substance. Of all the cultures, those grown in a solution containing 2 per cent. dextrose exhibited the most marked amount of the port-wine-colored material; that is, more cells contained this substance, practically all of the fan-shaped processes had regions which were stained port-wine color, and the color was deeper in tone and did not fade as rapidly as in the normal cultures. Saliva placed upon cultures in 2 per cent. dextrose prevented the appearance of the port-wine color, just as it did in the normal cultures. In these experiments with different amounts of dextrose it was impossible to predict whether a given cell would show the port-wine color in

the presence of iodine. The cytoplasm was not characterized by any structure indicative of this substance, but appeared the same in all of the cells. The processes of the mesenchyme cells had the peculiar lighter areas which in some cases became port-wine colored and in others remained pale yellow.

SUMMARY.

The cells of *Fundulus heteroclitus* grown in tissue cultures contain some substance which behaves in the same manner as does glycogen in the presence of iodine. It is possible that this substance may be glycogen.

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